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Time to speak out against cosy bilateralism

A YEAR and a half ago the governments of the United States and the Soviet Union declared that from March 1976 they would refrain from test-firing nuclear devices with yields of more than 150 kilotons. Atmospheric testing of devices of all sizes has been banned since 1963 (except that France and China never acceded to the treaty) but there has been a marked lack of enthusiasm among the nuclear powers for constraints on underground weapons tests. Now it looks as if even this modest bilateral measure may fail to materialise.

Testing of warheads has never been absolutely vital to a nuclear programme but there are obvious benefits in the regular, full scale firing of devices to try out new ideas. Weapons laboratories would find a total ban, if not unbearable, at least an inconvenience, and up to the present this has prevented really serious talk of a comprehensive treaty, in spite of regular pressure from non-nuclear powers who have seen the superpowers busily involved in 'upwards' proliferation while urging stringent controls against 'sideways' proliferation. For many years the vagaries of seismology were used to rule out a treaty—how could one go into an international agreement without adequate monitoring for violations? It will always be possible to deploy such an argument, of course, by insisting on lower and lower detection levels, but in recent years it has become clear that seismic monitoring capabilities are beginning to settle at a level at which a massive (and most unlikely) investment would be needed for further major advance.

When it became clear that the superpowers, under pressure to show at least some concern for the non-nuclear powers at the 1975 Non-Proliferation Treaty conference, were talking about a limited restriction, it was widely assumed that agreement would be reached on a level of about 50 kilotons (with which seismology could easily cope). When the figure of 150 kilotons was announced, there was general astonishment that anything so weak could seriously be proposed as an arms control measure. In hindsight, anyone who had seen the multi-lateral debates of the 1960s about strong arms control treaties give way to the bilateral SALT talks with their conveniently high ceilings should not have been surprised that the treaty had been designed to minimise inconvenience to the contracting parties. And the run-up to the treaty has allowed ample time for the testing of

larger devices—time which has been well used.

Unfortunately, two serious and entirely predictable problems have hampered the bilateral technical discussions thus far. The first is the difficulty in defining yield with any precision on the basis of remote measurements (the Soviet Union has consistently refused the United States access to any instrumentation on Soviet soil). The geological complexity of test-sites and the strange focusing and defocusing of seismic waves makes for uncertainties in yield determination of a factor of at least two, and the extensive East-West interchange of data in recent months does not seem to have brought much clarification.

The second problem is how to treat peaceful nuclear explosions. They have become very popular in the Soviet Union, which is thus unwilling to talk about even a 150-kiloton ceiling for its peaceful operations. If any doubted that warheads could be tested under a peaceful guise, India will have disabused them of that illusion. Ideally the devices would be supplied through an international agency from a stockpile, but if the International Atomic Energy Agency (IAEA) in Vienna has been approached to fulfil such a role it is being kept very quiet.

The omens are not good for the removal of these obstacles by March, but it is an election year in the United States and this tosses in many imponderables. If President Ford has to fight off a serious challenge from Mr Reagan he will probably wish to show he can be tough in foreign affairs—hence, no blind eye in treaties with the Russians, especially since Dr Kissinger has been so criticised for blind eyes to alleged SALT violations. But if President Ford wins the primary, a gesture of statesmanship before the election might well capture the public imagination (as President Kennedy found to his surprise after signing the Partial Test Ban Treaty).

Meanwhile the other nuclear and non-nuclear powers have to decide whether to raise a fuss in their forthcoming talks in Geneva about this cosy bilateralism. Some countries will. But how about Britain? Is it not time to drop the convention of not speaking against American initiatives, and to expose the treaty as a sham? Many people in high places in the United States might even be glad to have an old ally refuse to endorse a footling measure. □



The EEC countries have begun planning a more coherent approach to the exploitation of their joint energy resources. **Ray Dafter**, Energy Correspondent of the *Financial Times*, reports

Examining Europe's energy equation

THE SO-CALLED energy crisis of 1973 and 1974 may, in retrospect, be viewed as a blessing in disguise for, if nothing else, it applied the minds of nations to something they had chosen to ignore for decades: the sensible planning of energy resources for the future. Cheap and plentiful crude oil, mainly from the Middle East, had followed supplies of cheap coal as the basic energy provider and it seemed that everyone had been lulled into a false sense of security. As Mr Frank Zarb, the US Federal Energy Administrator, pointed out in London recently, the industrialised world had paid dearly for its mistake.

The first world conference on energy and raw materials, held in Paris in December last year; the establishment of the International Energy Agency (IEA) by the OECD; the bid for a common energy policy within the EEC—all these are signs of a change in attitude to energy. The fact that the Paris conference produced very little in the way of solutions to the international problem is hardly surprising; the issues are both complex and fundamental. Similarly, it is understandable that the IEA and the EEC are making heavy weather of finding common energy policies; after all, member states are only just beginning to get to grips with their individual plans.

But progress is being made. The EEC Nine, for instance, have already agreed on restrictions on the use of oil and gas in power stations and on the maintenance of minimum stocks of fuel in case of another energy emergency. They are now firmly committed to an energy conservation policy and to a programme to replace imported oil with alternative fuels. It is anticipated, for instance, that even if the EEC economy grows at 4% a year over the next decade, conservation measures should keep the growth of energy requirements down to no more than 3%: a saving of perhaps 250 million tonnes of oil equivalent in 1985. (Last year energy consumption in western Europe was down some 15% on earlier forecasts although it is difficult to measure just how much of this was due to deliberate conservation rather than the general economic recession.)

New agreements also emerged from the stormy EEC summit meeting in Rome early in December last year and

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Oil: 'Already there is a feeling in the industry that the plums of the North Sea have been picked'

these could well prove to be the cornerstones of future European energy policies. For a start there now seems to be widespread acceptance, both in Europe and in the wider IEA, that there should be a minimum safeguard price for oil—in essence a safety net for producers like the UK and Norway should world prices tumble.

Second, Mr Harold Wilson's apparently ignominious stand-down from his demand for a separate seat at the Paris energy talks did persuade France to back an oil-sharing policy in times of crisis: surely a vital principle in any unified energy policy.

Now comes the task of putting shape and meaning to these general policies. The European Community has, as a basic aim, the intention of cutting its dependence on oil imports from the present 60% of energy requirements to around 50% and possibly 40% by 1985. Here the North Sea must make a timely and important contribution (which was why Britain was seeking its separate seat at the energy talks in the first place). By 1980 Britain should be producing between 100 million and 130 millions tonnes of oil a year, making it the only country in the EEC self-

sufficient in energy. By 1985 Britain's North Sea oil could account for around 45% of the Community's total energy output; put another way North Sea oil and gas could, by 1985, be satisfying up to 12 to 15% of the Community's energy needs. In addition, the North Sea could be providing a privileged source of imports from the Norwegian sector worth perhaps a further 5–7% of needs.

This is where the importance of a minimum safeguard price comes in. By 1980 some £5,000 million is likely to have been spent on oil exploration and development in the UK sector of the North Sea. This money (plus a great deal more on ancillary facilities) is being invested on the assumption that oil prices will remain high and provide a reasonable return. If for some political reason Middle East producers decided to slash the price of their oil—and their production costs are a fraction of those in the hostile North Sea—then Britain and the exploration companies will find themselves in deep trouble. As it stands, the 18 members of the IEA have agreed in principle to a floor price of \$7 a barrel, more than \$4 below the present market price but sufficient, in the Agency's eyes, to protect the development of alternative sources of energy.

And this is what it is all about—reducing the industrialised world's dependence on OPEC oil and encouraging the development of other energy providers. Here, the North Sea provides a useful stop-gap, but it would be foolhardy at this stage to over-emphasise its long term importance. Even on British Department of Energy estimates, reserves from proven fields are likely to last for only 10 to 20 years, though, of course, new fields are being discovered at an encouraging rate.

Professor Peter Odell, Director of the Economic Geography Institute at Erasmus University, Rotterdam, and an often-quoted authority on energy matters, feels that present and future trends in the North Sea offer "the possibility of a long term supply of indigenous oil capable of bringing the dependence of western Europe on foreign oil down to almost a derisory level."

Undoubtedly new commercial fields will emerge in the North Sea over the

next decade (a new licensing round in the British sector is planned for later this year). How commercial they prove to be will depend on their size, the ease of recovery and the closeness to existing fields (which should help transporting costs), as well as on the price of oil. Already there is a feeling in the oil industry that the plums of the North Sea have been picked.

In the short to medium term, oil and natural gas must inevitably play a dominant role in the EEC's energy use—around 64% of total use if the Community's plans come to fruition, a large proportion of these hydrocarbons coming from the North Sea. The importance of natural gas should not be underestimated—the EEC hopes to obtain at least 175 million tonnes of oil equivalent from member countries by 1985; if possible some 225 million tonnes. But what happens when these valuable resources start to dry up? It would be fanciful to predict that some of the more colourful ideas will fill the gap. True a lot of research is going into harnessing energy from waves, tides, the wind and the Sun. On paper many of these possibilities look attractive. For instance, the energy theoretically available from waves is immense—the equivalent to about 70 kW per metre on the Atlantic-facing coastline of Britain alone. The sheer cost and technical problems put such schemes well into the future, however.

Even if one adds in geothermal heat, which has received so much publicity recently, it is unlikely that these newer methods of raising energy will provide more than 6–8% of requirements by the end of the century—at least this is the conclusion of a Department of

Energy 'think tank' (Energy Technology Support Unit) set up in June 1974.

This, then, leaves nuclear power and the 'old faithful', coal, to fill any gap left by a fall-off in oil or gas; or at least to take up much of the energy growth over the next 25 years or so. In some ways this is a daunting prospect, for in Britain this implies an expansion by 20 times of the country's present nuclear capacity by the year 2000; daunting because it poses the problems of technology (which type of reactor should be used), lack of indigenous uranium resources, and public attitudes (the row in the UK over nuclear waste disposal cannot be ignored).

Looking further afield, on present projections nuclear power would need to provide the EEC with 16% of its energy requirements if the Community is to reach 60% self-sufficiency by 1985. This is a big leap from the 1.4% share provided in 1973. The more optimistic projections for the use of nuclear fuel put the amount of installed EEC capacity at 200,000 to 225,000 MW (electrical) by 1985 whereas even the ambitious national plans of member countries go no further than 160,000 MW.

All these problems highlight the folly of writing off the coal industry as outdated and unloved. The coal industries in both the UK and Germany are likely to be given a boost over the next few years. Britain, for instance, has just begun to plan for an expansion programme that could take its output back up to 200 million tonnes a year by the end of the century. (In the past decade output has fallen from that



*Europe's Energy Commissioner,
Henri Simonet*

level to around 130 million tonnes a year.)

World resources of all types of coal have been estimated to be about 11 million million tonnes of which 700,000 million tonnes are known to be economically recoverable. But, coal or geothermal power, it is too early to assess with accuracy the relative importance of different forms of power even over the next decade. Policies are still emerging. But at least the industrialised world has learned the hard way that it must make the most of all available resources. It must be efficient in its use of energy, but above all it must be flexible. □

Turkish poppy industry

Straw cogs

The results of Turkey lifting its ban on the cultivation of opium poppies may surprise the sceptics, argues Peter Collins

THE Turkish Government seems to have scored a major success with its programme to control opium poppy cultivation and hence the illicit production of opium for the world's black market in drugs. Reporting on a visit to the poppy-growing areas at the harvest season, a United Nations official responsible for advising the Turkish government said that "no evidence could be found of opium production in Turkey in 1975". This should reassure those who may have had doubts about the Turks' ability to carry out their intentions, and confound certain United States senators and others who in 1974

foresaw America being flooded with heroin (the most dangerous opium derivative) when the Turks lifted their complete ban on opium poppy cultivation—a ban imposed largely because of pressure from the United States.

An important factor has always been that the Turkish farmers in the areas concerned have never grown poppies primarily as a source of opium, but rather for the seed and the oil derived therefrom which has traditionally been used as a normal part of their way of life. The Turkish government has prevented disruption of the local farming system by allowing the farmers to grow the poppies, though only on registered, and limited, areas. At the same time it has forbidden the lancing of the fruiting capsules, which must be done to get the opium. By harvesting the poppy straw (as the rest of the plant is called) as a monopoly, the government has also been able to continue supplying the world's pharmaceutical trade with the

raw material from which codeine, and hence various other materials, are extracted. All this has helped avoid a world shortage of codeine as well as cut off a potential source of illicit heroin.

No one expects a major programme like this to be a total success immediately. At least one of the problems raised could hardly have been foreseen. During an inspection tour of the growing areas, the UN team found that the government measures were if anything being too harshly applied. The areas that farmers had been allowed to sow were laid down in hectares, and a considerable number of farmers were found to have sown more than the area for which their licences allowed. The local farmers, it seems, think in terms of the dunum, an area which is actually slightly larger than the decare (1/10th of a hectare). Although this was a genuine mistake, understandable in a country where almost every region has

a local system of weights and measures, the law was extremely harsh on these men. Nor could any respite be obtained for them when the UN team pointed out that the excess area planted was in fact very small, and that officialdom as much as growers had made a mistake. The official argument has been that the arrest of the offenders will emphasise that the government really does mean business, and that to release them will bring the law into disrepute. Each must wait till the law has run its course; but if he can show that a mistake was genuinely made, he will eventually be released and granted a licence to grow poppies again, which would not otherwise be the case.

Meanwhile, the area any one farmer may cultivate has increased to a maximum of 5 decares from the permitted area in 1975 of 2 decares, which many farmers considered was too small to

be worthwhile. But it looks as though most of the farmers who have suffered arrest will not have had their cases heard in time for this year's sowing.

As far as the mechanics of the control system are concerned, the government programme seems to have been well organised and implemented. A number of 5-man teams were set up to inspect and control cultivation, and they are backed by 80 teams of gendarmes for detailed inspection. Vehicles for these teams have been provided through UN funds, and the same source of finance is being used to set up a telecommunications network between such major centres in the poppy-growing area as Afyon and Konya and the mobile inspection teams.

Other UN assistance is directed towards providing equipment for laboratories in Turkey for morphine

detection, carrying out training in this field at the Narcotics Laboratory in Geneva and training law enforcement personnel. The funds have also been used initially to underwrite a good guaranteed price for farmers. In 1975 they received 17 Turkish lira a kilogram for poppy "straw" and retained the seed which is their main interest. Although research is in hand to evolve more productive varieties of poppy in terms of both seed and morphine content, it is also hoped that eventually more farmers will turn to other crops, in accordance with official plans for the overall development of the areas concerned. Finally, plans are well advanced, again with UN advice and assistance, for the establishment within Turkey of a processing plant to handle the poppy straw which is now the main export product of the former opium-producing region. □

THE energy plan proposed last summer by the Minister for Industry, Sr Donat-Cattin, was approved on December 23 by the CIPE, the inter-ministerial committee for economic programming. The crux of Sr Donat-Cattin's plan is to supplement the three nuclear power stations already in existence with twenty or more by 1985, which will double the country's electricity production capacity.

In the heated debate over the plan this autumn, one of the most frequent criticisms has been that Italy will simply replace its dependence on the oil-producing countries with one on the uranium-producing countries, without gaining anything. It is also felt that the tremendous increase in capacity, which assumes growth of 4-5% a year, is unnecessary. The electricity board (ENEL) has countered this by pointing out that in the sharp recession of 1975 consumption decreased by only 1% of 1974 consumption, and claims that its plans are flexible enough to be adjusted in the future if necessary.

The US companies Westinghouse and General Electric are to build the 1,000 MW water-cooled reactors, and this has not pleased the Italian trade unions. Italian companies would, in fact, have been capable of doing a large part of the construction work, greatly relieving the balance of payments burden and the unemployment problem. And of course there are the worries about the safety of the US reactors.

A miniature and more furious version of the national debate has been going on in Lombardy, where Sr Donat-Cattin wants sites for two of the reactors to be found. The

regional government wants only one reactor. Instructed by the central government to find one site on the Po near Mantua and another near Lake Como, it has missed the deadline for doing either. The central government now has to choose.

Letter from Italy

from Gillian Boucher



● What elsewhere has been reported as Italy's blocking of the EEC project for nuclear fusion research has been seen in Italy as resistance by the other EEC countries to the Italian site for the project, Ispra. The EEC Commission felt Ispra to be the most suitable site for the experimental thermonuclear plant, the Joint European Torus (JET); led by the Minister for Science, Sr Pedini, to believe that the decision was virtually final, Italians were startled by the inability of the research ministers to agree about it when they met in Brussels in December.

As enumerated by the Commission, the advantages of Ispra over its competitors—the chief of which is

Culham in the UK—include its connections with Euratom, abundant electricity, available skill, experience of safety measures, and a pleasant environment (on the shores of Lake Maggiore) with an international school. Fears that work may be interrupted by labour problems seem exaggerated; Ispra has suffered industrial action in the past, but in 1975 only 34 hours were lost through strikes. The Euratom link, however, is not an entirely positive attribute: Ispra has constantly suffered from a lack of proper organisation and last summer faced a crisis which only a special grant of 2,000 million lire from the EEC averted. If it does not win JET its future looks extremely uncomfortable.

● After centuries of shooting anything that flutters or cheeps, the Italians have suddenly become wildlife-conscious. A bill on hunting has just been approved by the Senate; when the Camera, or House of Deputies, has approved it, it will provide general principles to guide regional governments in drafting their own rules.

According to the bill anyone who wants a gun licence will in future have to pass an examination on the applied biology and zoology of hunting, the law on hunting, firearms and how to use them, and conservation. Italians wishing to shoot will be required to take out third-party insurance, a protection which can hardly make them more carefree about where they point their guns than they are at present. Wildlife reserves will be established, and what may be shot where, when, and in what numbers will be specified in detail.

correspondence

The Loch Ness Monster

SIR,—In their recent article entitled "Naming the Loch Ness Monster" (*Nature*, December 11) Scott and Rines propose formal generic and specific names for a rhomboidal object photographed in Loch Ness. They fail, however, to demonstrate with any conviction that the object is animate, that it shares anything with the later photograph showing two very differently shaped images, or that there is any basis whatever for their suggestion that it represents a species of reptile.

One of the great achievements of eighteenth century zoologists was to devise a disciplined system for the description and naming of animals, one result of which was effectively to distinguish between the real and the mythical animals of earlier writings. The code of nomenclature that has been developed over the succeeding years has been very carefully designed to adjudicate only with regard to the choice of names, thereby avoiding any restriction of freedom in the interpretation of zoological evidence. The onus is therefore on authors and editors to maintain standards of description and rational argument to prevent a return to the days of uncritical mythology.

Readers of *Nature* might reasonably expect an article presenting and interpreting original taxonomic data to have been subjected to the normal refereeing process. The evidence presented for the existence of *Nessiteras rhombopteryx* as a new species of animal falls far short of any normal standards expected in taxonomic zoology, even allowing for the preliminary nature of the report. No details are given of the 'optical data' by which the sizes of the objects were determined, nor of the technique by which the first two photographs were determined, nor of the technique by which the first two photographs were 'computer-enhanced'. No mention is made of controls showing how familiar objects appear on film and sonar traces under the same conditions. These will presumably be included when the observations are published in more detail, but meanwhile it is inconceivable that the application of a name in these circumstances can serve the authors' objective of promoting the conservation of any large animal that might subsequently be found in Loch Ness.

Biologists daily encounter pheno-

mena that they cannot identify or explain. It happens every time a field ornithologist fails to identify a distant bird. He will normally prefer to explain his failure in terms of the limitations of his expertise, the poor atmospheric conditions or the extreme distance, rather than jump to the conclusion that it must be an undescribed species of bird. This analogy is very relevant to a great diversity of so-called unexplained phenomena in Loch Ness.

Zoological taxonomy and scientific publication in Britain have both achieved high reputations. It is a pity to jeopardise these reputations for no good cause. It would be an exciting day for all zoologists if convincing proof were to be produced of a new large animal in the zoologically best explored country on Earth. This paper is unlikely to persuade the scientific community that that day has arrived although it may well serve to mislead the layman into believing that it has.

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Sir Peter Scott replies:

G. B. Corbet, referring to a rhomboidal object shown in two photographs in the article "Naming the Loch Ness Monster", claims that its authors "fail to demonstrate with any conviction that the object is animate" by which no doubt he means that he is not convinced. Others with equal claims to scientific objectivity have expressed an opposite view, being impressed by the slight difference in the configuration and the orientation of the flipper shown by the pictures, taken one minute apart by a fixed camera on the bottom, which clearly shows that the structure cannot be rigid.

Dr Corbet chooses to ignore the simultaneous sonar evidence with its clear indication that moving objects of large size were present at the time the photographs were taken. He says we have failed to demonstrate "that there is any basis whatever for the suggestion that it represents a species of reptile". We explain that we believe the flipper belongs to a vertebrate animal and that no known aquatic mammal has such a limb. It has not been possible to attribute the shape to any known fish or amphibian; on the other hand, the shape conforms rather closely with the limbs of certain fossil reptiles. In spite

of his analogy which refers to "an undescribed species of bird", it seems unlikely that Dr Corbet would prefer to attribute the Loch Ness Monster to that class. The actual quote from the article is "the inclination is to view it as reptilean".

The objective of the paper was quite clearly stated in the preamble: "Better to be safe than sorry; a name for a species whose existence is still a matter of controversy among many scientists is preferable to none if its protection is to be assured". I do not know what particular expertise Dr Corbet may have for asserting that the objective will not be achieved. Meanwhile it seems sad that he is working so hard to prevent it.

* * *

SIR,—The publication of Scott and Rines and the photographs in the national press indicate that there may be a plesiosaur-like reptile inhabiting Loch Ness. It is exceedingly difficult to envisage how a former tropical marine reptile could endure the cold waters and harsh environment provided by a small lake in Scotland. Since Loch Ness did not exist until some 12,000 years ago, one is faced with the problem of the survival of 'Nessiteras' for a period of 64 million years in a world where its former ecological niche had been occupied by modern cetaceans and pinnipeds.

A certain amount of research on the functional anatomy of plesiosaurs has been undertaken and the results widely reported*. There were two major types: the long-necked which fed on fish in the surface waters, and the streamlined large-headed forms which fed on cephalopods and were capable of diving to depths of 300 m. The postulated shape of 'Nessiteras' is reminiscent of the non-diving surface living variety; the postulated behaviour is of the large-headed short-necked forms. The evolutionary history of both groups of plesiosaurs can be traced for a period of 150 million years with very minor changes being recorded. It is inherently improbable that from such a stock this strange mixture of both groups would suddenly emerge.

The three key pieces of photographic evidence, which purport to show the neck and part of the body, the right hind flipper and the head, deserve to be analysed in the context of the accumulated knowledge of both living

and extinct aquatic reptiles and other water-dwelling air-breathers. The only way that the neck and body picture could be restored to form a plesiosaur would be to cover a skeleton with skin, but with the musculature reconstructed a different shape would be produced. The appearance of a truncated 'limb' and its negative on the opposite side of the 'body', together with other rectangular areas, would seem to exclude any possibility of this structure being reptilian.

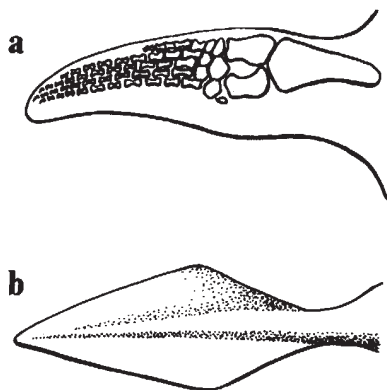


Fig. a Hydrofoil limb of *Plesiosaurus* to show extent of soft tissues (after Robinson 1975).

Fig. b Oar forelimb of *Nessiteras* to show axial 'skeleton' (after Scott and Rines 1975).

The correct shape of plesiosaur flippers is rarely illustrated, but it has been known since Dames described skin impressions in 1895. The bones were situated along the leading edge with a tapering fleshy trailing edge; the plesiosaur limb was hydrofoil-shaped (Figs a and b). The limbs of plesiosaurs functioned in the same way as those of marine turtles, penguins and sea lions. The type of fin described by Scott and Rines is not known to exist in any marine vertebrate to our knowledge. It is not a hydrofoil but instead is oar-shaped. There is a central axis and the distal end tapers to a point thus reducing drag. It is inconceivable that an animal with efficient hydrofoil limbs should dispense with them for inefficient oars.

The heads of marine reptiles have their nostrils situated immediately in front of the orbits (the crocodiles are the exception to this but they have achieved the same functional end by evolving a secondary palate). Furthermore, the heads are streamlined. In contrast to this, the photograph of the Loch Ness head has terminal nares with the nasal region being clearly marked off from the orbital by a pronounced ridge or step. There even appear to be horns growing from the frontal region. There is no hint among any group of reptiles of such quasi-mammalian contours.

The evidence claimed to establish

the existence of an aquatic reptile '*Nessiteras rhombopteryx*' allows of an alternative and more logical interpretation.

The 'body-neck' photograph could be of the prow or stern of a Viking ship; the positive and negative projections would be transverse cross-beams of the hull; the longitudinal rectangle would be one of the main planks. It is perhaps worth noting that there are records of Viking raids on ancient settlements in the region of Loch Ness, for example Iona.

Mr Sheridan has already pointed out (reported in *The Times*) the similarity of the 2m long right hand fin to the steering rudder of Viking ships, which is always situated at the starboard stern (posterior right-hand side). Finally the head photograph is exceedingly similar to the dragon heads with which the Vikings embellished the prows of their vessels (and royal furniture). The Loch Ness head would appear to be generically related to the Oseberg head in Oxenstierna's *The Norsemen*.

The features attributed to the new taxon *Nessiteras rhombopteryx* are inconsistent with the anatomy and inferred functioning of any group of extinct reptile. The conclusion seems inescapable: Scott and Rines have discovered the remains of a Viking ship and have mistakenly interpreted them in terms of a living organism.

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* Watson, D. M. S., *Proc. zool. Soc. Lond.*, 885 (1924); Tarlo, L. B., *Palaeontology*, 1, 193 (1958); *ibid.*, 2, 39 (1959); *New Scientist*, 1414 (1960); Newman, B. H., and Tarlo, L. B., *Animals*, 10 (2), 61 (1967); Halstead, L. B., *The Pattern of Vertebrate Evolution*, 132 (Oliver and Boyd, Edinburgh, 1969); Halstead, L. B., and Middleton, J. A., *Bare bones—an exploration in art and science*, 29 (Oliver and Boyd, Edinburgh, 1972); Robinson, J. A., *N. Jb. Geol. Paläont. Abh.*, 149, 286 (1975); Halstead, L. B., *The evolution and ecology of the dinosaurs*, 68 (Peter Lowe, London, 1975).

Sir Peter Scott replies:

Your correspondents Halstead, Goriup and Middleton argue interestingly that plesiosaurs were either long-necked and lived in shallow water eating fish, or short-necked and dived to 300 m to catch squids. They claim that the behaviour of *Nessiteras* (of which we know very little) living in a loch that is 300 m deep, but being long-necked, is "a strange mixture of both groups" and therefore "inherently improbable." But in many animal orders whose evolution displays adaptive radiation we find primitive types surviving among the more advanced. To discover features of two known groups combined in one species does not necessarily postulate reticulate evolution.

I agree with your correspondents when they say "it is inconceivable that

an animal with efficient hydrofoil limbs should dispense with them for inefficient oars." But how do we know that the ancestors of *Nessiteras* ever had efficient hydrofoil limbs, and who is to measure the efficiency of their diamond-shaped flippers against the functions they have to perform? Evidently it is adequate for their mode of life.

It may be worth remembering that scores of species of some 53 genera of plesiosaurs are known to science from their fossil bones. In none of them is the skin contour of the head recorded in the stone, and only two examples show the skin contour of the flippers. In many cases the shapes of both birds' wings and fishes' fins vary widely within a single order.

Nor should we forget the processes of convergent evolution. Our paper describing *Nessiteras* said of the flipper "the inclination is to view it as reptilian." Nowhere in the paper was the name plesiosaur used.

The theory that the photographs depict the remains of a Viking ship does not fit the facts, even if the vessel were to be drifting round in midwater like a submerged Flying Dutchman. It is quite impossible, within a number of limiting circumstances, for the head photograph to be a stationary object attached to, or resting on the bottom. These limitations include the geometry of the camera, its strobe-flash equipment and the rope from which it was suspended from the boat, the distance and nature of the bottom below, and the turbidity of the water. On the other hand the Dragon head from Oseberg which they show may, in spite of its mammalian connotations, perhaps have been influenced by monsters well known to the Vikings.

The interpretation of the two pictures of the flipper as a rudder of a Viking ship is perhaps a measure of the inadequacy of modern newsprint reproduction. In the enlargements of the computer-enhanced photographs it is especially interesting that, in the interval of one minute between exposures in a camera standing stationary on the bottom, the flipper has changed shape and orientation. The changes are entirely consistent with the movement of an animal's swimming limb, and could not conceivably have happened if the object had been fixed and solid. These photographs were taken simultaneously with the moving objects shown in the sonar trace published with our article, which seem to have been conveniently ignored by your correspondents. They end with "an inescapable conclusion" from which they might do well to escape after all. If they are interested in Viking ships they will have to go elsewhere to find them.

news and views

Terminators and attenuators

from Andrew Travers

POSITIVE control of early gene expression in phage λ requires the function of λ N protein. This regulator permits the transcription of λ early genes yet its sites of action are clearly distal to and distant from the early λ promoters. There is, however, a striking correlation between these sites and transcriptional terminators whose efficient function *in vitro* requires the protein ρ . From this evidence Roberts (*Nature*, **224**, 1168–1174; 1969) proposed that N antagonised ρ function and thus allowed these termination signals to be overridden with a consequent extension of the RNA chains initiated at early promoters.

A further insight into the role of ρ *in vivo* came with the observations that there are ρ -sensitive terminators within the *gal* operon and that mutant *gal* operons containing polar insertions are also polar *in vitro* in the presence of ρ (de Crombrughe *et al.*, *Nature new Biol.*, **241**, 260–264; 1973). This suggested that natural polarity of bacterial operons might be a consequence of ρ function. Another example of transcriptional polarity, that induced by nonsense and frameshift mutations, can be suppressed by second site mutations within the *suA* gene. These mutations, which are recessive to the wild type allele, partially relieve the polar effect of the original mutation without altering the termination of protein synthesis induced by that mutation (Morse and Primakoff, *Nature*, **226**, 28–31; 1970). This parallel between the *in vitro* effect of ρ in inducing polarity and the *in vivo* effect of *suA* mutants in relieving polarity raised the possibility that ρ might be the product of the *suA* gene.

Earlier this year this possibility received strong support from observations of Richardson *et al.* (*Proc. natn. Acad. Sci. U.S.A.*, **72**, 1725–1728; 1975). They found that ρ factor purified from a strain containing the *su78* mutation in the *suA* gene has altered termination function. This ρ factor fails to terminate transcription at some sites that are efficiently recognised by ρ factor purified from an isogenic wild type strain. Moreover, a second

suA mutation reduces the recovery of ρ activity from crude extracts by more than 90%. These results do not in themselves prove that ρ is the product of the *suA* gene, since mutation in that gene could indirectly affect ρ activity. They do show, however, that termination factor activity is altered in strains containing a polarity suppressor thus again implying that ρ is involved in transcriptional polarity.

The connection between ρ function and polarity allowed two predictions to be made. First that polar mutations in bacterial operons might be overcome by active λ N protein. Second that λ N gene mutants which are unable to grow because of their failure to produce sufficient λ early mRNA should themselves be rescued by polarity suppressors. Both predictions have now been verified. N gene function overrides polar mutations in the *gal* and *trp* operons (Adhya *et al.*, *Proc. natn. Acad. Sci. U.S.A.*, **71**, 2534–2538; 1974; Franklin, *J. molec. Biol.*, **89**, 33–48; 1974; Segawa and Imamota, *J. molec. Biol.*, **87**, 741–754; 1974) but only when transcription is initiated at a λ promoter *in cis* to the bacterial genes. This implies that N function requires a phage specific site in order to alter the properties of the transcription complex. The experimental approach to test the second prediction was the direct selection of bacterial mutants which specifically suppress λ N mutants (Brunel and Davidson, *Molec. gen. Genet.*, **136**, 167–180; 1975). These bacterial mutants, designated *sun*, map very close to the known map position of *suA*. Moreover the *sun* mutants are themselves able to suppress polarity in a bacterial operon, albeit only weakly. Conversely one *suA* mutant exhibits a *sun* phenotype. All these results suggest that *sun* and *suA* are allelic. If the only major role of N protein is to antagonise ρ , *suA* gene function is thus again linked to ρ activity.

To what extent is the antagonism of transcriptional termination by N protein a specific example of a more general mode of controlling gene expression? Two recent findings suggest that the phenomenon may be wide-

spread. Roberts, together with Sklar *et al.* (*Proc. natn. Acad. Sci. U.S.A.*, **72**, 1817–1821 and 3300–3304; 1975), has provided evidence that the synthesis of λ late mRNA may be controlled in a similar manner to early λ mRNA. They show that a transcription terminator occurs between the promoter for late gene expression and the late genes themselves. This terminator results in the *in vitro* production of a small RNA species about 200 nucleotides long. Late λ mRNA synthesis is under positive control of gene Q and Roberts proposes that the Q gene product acts in a manner analogous to N protein. If this is so it follows that anti-terminator proteins may differ in specificity.

Nor is the positioning of a terminator closely distal to a promoter exclusive to λ . Bertrand *et al.* (*Science*, **189**, 22–26; 1975) have described a strikingly similar structure in the *E. coli trp* operon. The existence of a terminator close to the *trp* promoter was first suggested by the properties of small deletions between the *trp* promoter-operator region and the first structural gene in the *trp* operon. Such deletions produce elevated levels of both *trp* mRNA and polypeptides yet the *trp* repressor-operator interaction is unaffected by the deletion. This effect is *cis*-dominant. Analysis of the RNA produced *in vivo* and *in vitro* from the region immediately distal to the promoter reveals a major RNA species 130 nucleotides long corresponding to the 5'-terminal sequence of the normal *trp* mRNA. *In vivo* the extent of termination resulting in the production of this RNA is regulated. In particular tryptophan deprivation results in relaxation of termination with a corresponding increase in the levels of *trp* mRNA and polypeptides produced. Bertrand *et al.*, propose that the terminator site may function to govern the fraction of initiated polymerase molecules that will transcribe the entire *trp* operon. The control is thus a quantitative modulation rather than a strict on-off switch and accordingly the authors suggest that this DNA signal for termination be termed an 'attenuator'. Other evidence indicates that similar atten-

uators may control expression of the *E. coli* *ilv* operon and the *S. typhimurium* *his* operon (Kasai, *Nature*, **249**, 523–527; 1974).

The parallel between the *trp* attenuator and λ terminators overridden by N function is strengthened by the finding that polarity suppressors allelic to *suA* partially relieve attenuation. The original *suA* mutants are, however, relatively inefficient in this respect. Again different alleles of *suA* differ in their effectiveness at suppressing polarity at different terminators. The ubiquitous association of *suA* mutants with a failure to terminate transcription coupled with the selectivity of different *suA* alleles argues that ρ may indeed be the product of the *suA* gene.

This strong suspicion is confirmed by two observations of Ratner (this issue of *Nature*, page 151). First, affinity chromatography analysis of ρ protein from different *E. coli* strains reveals that different *suA* mutations affect ρ in disparate ways: one destabilises ρ , a second apparently increased the molecular weight of the polypeptide, while others which are presumed to be missense mutations overproduce ρ . Finally by making use of a molecular weight difference between ρ isolated from different *E. coli* strains Ratner shows that the ρ gene is tightly linked to the *suA* locus. Together these experiments make it almost certain that the *suA* locus defines the structural gene for ρ and not a modifier of ρ protein.

Solar seismology

from Nigel Weiss

THE detection of normal modes of vibration in the Sun provides the first proper check on the theory of stellar structure and evolution. By astrophysical standards, this theory is well established and precise: it predicts the properties of main sequence stars and the qualitative features of their later evolution. These successes are obtained after calibrating the theory to fit the mass, radius, luminosity and age of the Sun, all of which are known. The single other measurement that could be used to check the theory was of the solar neutrino flux. Unfortunately, this is an order of magnitude less than theory had predicted. In the last six months, however, three independent groups have announced the discovery of solar oscillations and the periods of these oscillations offer a means of probing the internal structure of the Sun.

About 10 years ago H. A. Hill set up the Santa Catalina Laboratory for Experimental Relativity by Astrometry, in Arizona, with the aim of measuring precisely the gravitational deflection of light by the Sun. To do so required an accurate technique for locating the edge of the solar disk. The SCLERA group were then able to measure the Sun's oblateness and to show that it was consistent with the observed rotation rate at its surface. In addition they discovered regular oscillatory displacements of the edge of the Sun with a period of about 48 min, together with nine higher frequencies of oscillation.

At the Crimean Astrophysical Observatory, A. B. Severny has for many years studied magnetic fields and velocities in the solar photosphere. He has also compared stellar magnetic fields

with the net field that would be measured if the Sun could not be resolved. In this issue of *Nature* (page 87), Severny, Kotov and Tsap report the result of comparing the line of sight velocity at the solar surface near the equator with that around the poles. This enables them to estimate the radial velocity and they have discovered regular oscillations, persisting for at least 7 months, with a period of 2 h 40 min $\pm$ 0.5 min.

This remarkable result has been independently confirmed (page 92) by a group from the University of Birmingham. Brookes, Isaak and van der Raay were attempting to measure the gravitational redshift from the Pic-du-Midi Observatory, using a resonance scattering method. Their results, also reported in this issue, show oscillations with a period of 2 h 39 min $\pm$ 2 min. The agreement is far too close to be fortuitous; the methods are so different that systematic errors have to be excluded. Thus the Sun seems also to be pulsating with a period more than three times greater than the longest period detected by the SCLERA group. (Hill's procedure was limited to periods less than about 1.5 h.)

In a third article in this issue (page 89), Christensen-Dalsgaard and Gough, from the University of Cambridge, attempt to identify the normal modes observed and relate them to those derived from theoretical solar models. Stellar pulsations can be classified as acoustic (p) or gravitational (g) modes. The fundamental mode of radial pulsation for a typical solar model has a period of approximately an hour, while the fundamental quadrupole mode has a period of 46 min. In general, the period of an acoustic mode

decreases with increases either in the number of internal modes or in the degree of angular dependence. The computed periods for p modes are in fair agreement with the SCLERA observations, though it is not yet certain how they are excited.

This leaves the problem of explaining the 2 h 40 min oscillation. The observers suggest that it is indeed a fundamental radial pulsation of the Sun. If so, this measurement would upset the established theory of stellar structure and, with it, many astrophysicists. Despite the anomalous neutrino flux, it seems unlikely that the accepted theory could be so far wrong. The theorists prefer to interpret this long period oscillation as a high order gravitational mode, for the g modes have periods that increase with the number of internal modes.

Christensen-Dalsgaard and Gough also review the exciting prospects opened up by these new observations. It should soon be possible to identify the modes directly and to determine their frequencies with great precision. These frequencies can then be used to establish the properties of the solar interior and, in particular, the variation of chemical composition with radius. Such combinations of exact measurements with detailed computations are changing the nature of solar physics. This inversion procedure is similar to that used in deriving the Earth's structure from the frequencies of its normal modes (of which over 1,000 are now known). The structure of the Earth had, however, already been established by seismic observations: in the Sun, though we think we know the relevant equations, the structure has not yet been probed. Solar seismology introduces a new precision into astrophysics. $\square$

Two more biologically active tetrapeptides

from A. J. S. Davies

THE search for chemically defined mediators of interactions between mammalian cells is an arduous process. The lymphocytologists, flushed with success at their identification of T and B cells, have been trying valiantly but, aside from a long list of somewhat risible acronyms (MIF, MAF, SMAF, LIF, LAF and so on) little of repute has yet emerged. The chalone concept has been put forward but is tending to founder on the lack of support consequent upon failure of chemical characterisation of the putative medi-

ators. Thymic hormones and transfer factor are part of this twilight world of 'things' which could be useful, but which would gain more credibility if their identities were convincingly established. As we become more aware of the complexities of cell effluents and become more able to place them in biological perspective then the search and the arguments as to worth and reality will become hotter. How nice, against a background of increasing confusion, to find simple chemically defined mediators of a cell interactional process.

Goetzl and Austen (*Proc. natn. Acad. Sci.*, **72**, 4123-4127; 1975) describe in their paper two sequenced tetrapeptides derived from lung tissue which have the capacity to attract eosinophils. It has been known for some time that eosinophil chemotactic factor of anaphylaxis (ECF-A) could be released from either guinea pig or human lung slices during immediate type hypersensitivity reactions, for example contact with rag weed pollen after passive sensitisation with anti-rag weed serum containing high IgE levels. Further it was found that the material released was preformed in basophils and mast-cell-rich organs. The release was modulated by the levels of intracellular cyclic nucleotides in a similar manner to the regulation of histamine release. ECF-A itself was more effective in attracting eosinophils than neutrophils in the rather artefactual chemotactic chambers used for these quantitations.

Goetzl and Austen caused release of ECF-A from human lung tissue by appropriate means or extracted it from the same kinds of lung. The lungs were surgical specimens and one wonders idly why they were removed. The ECF-A was shown to consist of two tetrapeptides the NH_2 -terminal amino acid of which was either valine or alanine. These two peptides were then synthesised as was the common tripeptide Gly-Ser-Glu. Both the extracted, released and resynthesised tetrapeptides were shown to have the same high levels of activity and to have a slightly preferential attractive effect on eosinophils compared with their effect on granulocytes. It was further shown that eosinophils which had been exposed to the synthesised peptides became incapable of migrating in response to ECF-A (neutrophils were similarly inactivated).

There are many things of interest in this work. Is the factor really as specific for eosinophils as its name indicates? Do the various lymphocytic regulators of eosinophilia operate by causing release of the factor or do they operate solely by potentiating the production of the appropriate antibody in order that an anaphylactic release

might occur? Are these tetrapeptides mediators *in vivo*? One also wonders what is the functional relationship between ECF-A and tuftsin, the phagocytosis-stimulating tetrapeptide (Nishioka, *et al.*, *Biochem. biophys. Res. Commun.*, **47**, 172-179; 1972). Tuftsin and ECF-A are dissimilar in sequence but the existence of at least three tetrapeptides which have biological activities in relation to granulocytes could indicate that many such molecules exist. □

The IPCS—a major advance in astronomical spectroscopy

from M. S. Longair

It is now over two years since the first spectra taken with the Image Photon Counting System (IPCS) developed by Alec Boksenberg and his collaborators at University College London caused a major stir in astronomical circles. Advanced electronic and television techniques had been applied to astronomical spectroscopy in a device which counts the numbers of photons in different wavelength ranges and stores this information in a computer. Since then a steady flow of papers containing important results obtained with the system have appeared, in particular two recent papers on the quasar 3C273 and the nucleus of the nearby Seyfert galaxy NGC4151 (Boksenberg *et al.*, *Mon. Not. R. astr. Soc.*, **172**, 289 and **173**, 381 respectively). Both sets of observations were made with the Isaac Newton 98-inch telescope at the Royal Greenwich Observatory, Herstmonceux.

In normal astronomical spectroscopy, the light dispersed by a diffraction grating is focused on to a photographic plate and the information is recovered by developing the plate and measuring features directly from the plate or from some form of isodensitometer tracing. In the IPCS, the dispersed light is focused on to the photocathode of an image intensifier of very high gain. Each photon registered by the image intensifier produces a "spot" of light at the output of the tube which is continuously scanned by a television camera. By electronic processing, this "spot" of light is recognised as a single photon appearing at a single point in the dispersed spectrum. The photons are binned into 1,024 separate wavelength channels spanning the wavelength range of the grating. The television scanning technique enables the infor-

mation on each arriving photon to be transmitted to an on-line computer which stores the information continuously in core. Thus the accuracy with which the spectrum can be observed is limited by counting statistics of the photons detected. In turn this is determined by the cathode efficiency of the image intensifier which in the best modern tubes is better than 20% in the blue.

In practice, the IPCS system is operated in a dual-beam mode, with the object in one aperture and a nearby blank region of sky in the other so that the sky background and the background dark current in the image intensifier can be measured directly. It is normal practice to make two sets of observations with the object and background apertures interchanged to compensate for variations in the sensitivity in different parts of the photocathode. Subtraction of the backgrounds is then trivially performed in the computer. Once the observations are completed, the observer can display the spectrum of the object on a television monitor and plot it on large scale graph paper. Additional facilities include provisions for smoothing the spectrum, removing the sloping continuum to measure precisely the centre wavelength of spectral features and so on. The observer can come away from the telescope with his spectra calibrated and ready for analysis. The spectra shown in the papers on 3C273 and NGC4151 illustrate the effectiveness of the sky subtraction technique, the sky lines having almost completely disappeared from the spectra.

There are many practical advantages in such a system which greatly simplify the process of taking astronomical spectra. The integrated spectrum can be observed "growing" in real time as the observation proceeds since the total counts can be continuously displayed on the television monitor. This is particularly valuable when the objects being observed are too faint to be seen visually through the telescope eye-piece and off-set guiding is necessary. Also as the information acquired is directly stored in the computer, there is no possibility of "forgetting to put the plate in" or "ruining the plates". The system is designed to study faint objects and with the 200-inch telescope, for example, the spectrum of a quasar of 20 mag can be obtained in about half an hour. If the rate of arrival of photons is too great the television scanning system saturates, but since many of the most interesting objects are very faint—quasars, radio galaxies, globular clusters in external galaxies—this is not considered by most astronomers to be a disadvantage.

Since it has come into operation the system has proved very reliable and a

negligible percentage of observing time has been lost through faults in it. A measure of the high regard which astronomers have for this device is the fact that it was used continuously on the 200-inch Hale telescope for a period of over 2 months last year and over 500 spectra obtained. At present it is the most powerful system of its type in the world.

The quality of the spectra displayed in the two papers in *Monthly Notices* speak for themselves. Besides the increased speed with which such spectra can be obtained, the main advantages lie in the determination of the absolute intensities of the lines and in the ease with which broad or weak features in the spectra can be recognised. For example, in the spectrum of NGC4151, over 80 narrow lines can be identified and absolute intensities are quoted for all of them. This much improved data base provides a new challenge to theorists for the construction of models of the line-emitting regions in complete nuclei. Perhaps more striking is the presence of very broad wings on the Balmer lines, the lines of He II and of Fe II. These are all permitted transitions and are much broader than the forbidden lines. The fact that no broad forbidden Fe II emission is observed indicates that the broad spectral features arise in regions where the particle density is greater than 10^7 cm^{-3} . This situation is very similar to that observed in 3C273 where the forbidden lines are either very weak or absent. In this respect 3C273 differs from most other quasars.

These papers represent the tip of the iceberg—hundreds more spectra await analysis in many different fields of astrophysics. Systems such as the IPCS will shortly be standard equipment on all the large optical telescopes; for example, Boksenberg and his colleagues are at present building a new version of the IPCS for use on the 4-m Anglo-Australian telescope. Thanks to the development of these devices, many very large scale projects which would have seemed quite impracticable 10 years ago are now within the capabilities of the new generation of telescopes. □

Madagascar issue settled

from Peter J. Smith

THE geographical position of Madagascar before the breakup of Gondwanaland has been a source of controversy for well over a decade. Before Wegenerian drift began, did this continental fragment lie to the north or

south of its present location? Or has it always been at its present latitude, with only a small eastward drift to take it away from the African mainland and create the Mozambique Channel? The confusion now seems to be over, as palaeomagnetism has provided the definitive answer (Embleton and McElhinny, *Earth planet Sci. Lett.*, **27**, 329; 1975).

The first person to say anything about the ancient position of Madagascar was Wegener himself who placed the island in the large coastal embayment of Mozambique, or about 4° south of its present position. Intuitively (which is simply to say that there is some worth in the Baconian rule of "conformable instances"), this is the obvious place to put it, although there are also some respectable geological arguments for this view as Flores (*Trans. geol. soc. S. Africa*, **73**, 1; 1970) has shown. On the basis of aeromagnetic profiles across the Mozambique Channel, Green (*Nature phys. Sci.*, **236**, 19; 1972) also supported a fit against Mozambique, in which he was followed, on other grounds, by Kent (*Nature*, **238**, 147; 1972).

On the other hand, Darracott (*Earth Planet. Sci. Lett.*, **24**, 282; 1974), taking account of the thin continental-type crust in the Mozambique Channel, concluded that Madagascar has always been at its present latitude but has drifted slightly eastwards. This was a slight variant of an earlier proposal from Flower and Strong (*Earth planet Sci. Lett.*, **7**, 47; 1969) that the island has remained stationary, although Wright and McCurry (*Earth planet. Sci. Lett.*, **8**, 67; 1970) felt that the evidence cited by Flower and Strong could not be taken to exclude eastward drift, arguing instead that Madagascar once lay against Mozambique.

The third and, on the face of it, more radical proposal is that Madagascar has moved about 15° southwards and slightly eastwards, in which case it would have originated against Kenya and Tanzania. This view was first put forward by Du Toit (*Our Wandering Continents*, Oliver and Boyd, Edinburgh, 1937) largely on the basis of matching lithologic units and later supported by Smith (A. G.) and Hallam (*Nature*, **225**, 139; 1970) on the basis of a computer fit of the 500-fathom contour. After carrying out a geophysical survey in the Mozambique Channel, Heirtzler and Burroughs (*Science*, **174**, 488; 1971) also concluded that Madagascar has drifted southwards.

Among the mass of conflicting geological and geophysical evidence, palaeomagnetic data is conspicuous by its absence. It is easy to be wise after the event, but an obvious way of deciding between the three hypotheses

would be to obtain a palaeomagnetic direction for rocks laid down on Madagascar before Gondwanaland split and compare it with African mainland directions for the same epoch. It is true that over a decade ago Nairn (*Overseas Geol. Min. Resour.*, **9**, 302; 1964) obtained a palaeomagnetic direction from the Karroo sediments of Madagascar, but no magnetic cleaning techniques were used and at that time there was no suitable African data for comparison.

Using modern methods, Embleton and McElhinny have now obtained a good palaeomagnetic pole position from Triassic-Jurassic sediments on Madagascar and have compared it, first, with the Triassic-Jurassic African pole and, second, with the African pole for the whole of the Mesozoic (possible because Africa has apparently remained fixed in position during this period). Irrespective of how the comparison is carried out, there is no significant difference between the Madagascan and African poles only if Madagascar was north of its present position during the Triassic-Jurassic. In other words, according to palaeomagnetic evidence there is little doubt that before the onset of Wegenerian drift Madagascar lay off the east coast of Africa adjacent to Kenya and Tanzania.

Having made this central point, Embleton and McElhinny go on to cite the geological and faunal observations in its favour. This evidence is convincing too, although no doubt some puzzles still remain. The fact is, however, that the grounds of argument have now shifted. Any geological 'anomalies' must now be reconciled with Madagascar's northern origin: they can no longer be regarded as convincing evidence against it. □

Immunoglobulins: constant sequences in the variable region

from Pamela Hamlyn

ANTIBODIES—the vast number of protein molecules which react specifically with foreign substances to trigger the immune response—are complex molecules all very similar in design. Each has two heavy (H) and two light (L) chains. Both H and L chains contain a constant (C) and a variable (V) region with regard to amino acid composition. Analysis of related L chains has shown that different amino terminal regions are associated with the same C region and has led to the suggestion that, unlike all other polypeptides, two genes are needed to code for one polypeptide chain. The number of C region genes

seems to be small but there is controversy over the number of V region genes. These genes code for the enormous number of polypeptide chains which confer specificity on the antibody molecules.

Theories to account for the diversity of the V region genes fall into two main categories. Either they suggest that there is a very large number of genes in the germ line encoding the many V regions, or that the number of genes in the germ line is small but it is amplified by somatic processes such as mutation, recombination or excision and repair with error of the DNA. Despite very many attempts in the years following the publication of these proposals no experimental data has been obtained which clearly establishes one theory over the other. Recent information on the V region of L and H chains indicates that variability is localised in interspersed hypervariable regions. The remainder of the V region (framework segments) show a remarkable preservation of amino acid sequences among related chains.

Capra and Kindt have described a model to account for these observations (*Immunogenetics*, **1**, 417; 1975). They propose that the interaction of at least three genes is required to form each immunoglobulin polypeptide chain, the relatively invariant portion of the V region being encoded by a limited number of germ line genes while the hypervariable genes would be coded for by a larger number of genes, perhaps 50–100. By some integration mechanism the hypervariable genes would interact with the basic subgroup genes to form the completed variable region which would then, by a further integration, link to the constant region to form the completed gene for the whole polypeptide chain. This model helps to reconcile some of the conflicting evidence used in support of the alternative theories.

Some recent experiments of Frieden-son, Tung and Nisenoff (*Proc. natn. Acad. Sci. U.S.A.*, **72**, 3676; 1975) could be used to lend support to Capra and Kindt's model. They isolated antibodies of the same specificity from nine individual mice immunised with the same antigen and used the H chain for amino acid sequence analysis of the first thirty or so N terminal amino acids. They found that the amino acid sequences were all identical. Previous studies of this framework region had investigated amino acid sequences in antibodies pooled from several mice so that occasional amino acid substitutions (arising from somatic mutations) would have been difficult to detect. These results therefore strongly imply that somatic mutations do not occur in the

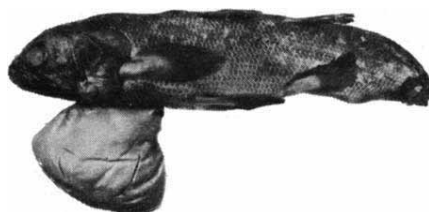
first framework segment and the authors suggest that this may be extrapolated to apply to framework regions in general. Sequence data on the remainder of the variable region will of course be necessary to see if this extrapolation is justified, and to see how the sequences in the hypervariable regions are related in different individuals. □

So the coelacanth does bear live young

from a Correspondent

A SHORT paper published in *Science* (**190**, 1105; 1975) by C. Lavett Smith, Bobb Schaefer, and James W. Atz of the American Museum of Natural History, New York, and Charles S. Rand of the Department of Biology, Long Island University, announces the discovery of five advanced young within the body of a preserved female coelacanth, *Latimeria chalumnae*. Their discovery finally settles a long period of uncertainty as to whether *Latimeria* lays eggs or bears live young.

Since its discovery off South Africa in 1938 the coelacanth has been slow to yield its secrets. It was not until 1952, following wide publicity by the late J. L. B. Smith (who described the first specimen), that a second specimen was captured, this time off the Comoro Islands. This revealed the true geographical range of the species to be this French possession in the western Indian Ocean, and since that date some 80 specimens have been captured—all by native fishermen—despite two expensive multinational expeditions to the Comoros. Most of these specimens have been retained in France, one of the exceptions, however, was the female 1.6 m in length and weighing 65 kg at capture in 1962 that is now in the American Museum of Natural History.



Coelacanth young with yolk sac

Despite this apparent wealth of material the key question as to whether these fish lay eggs or give birth to live young remained unanswered. Millot and Anthony (*C. r. Acad. Sci. Paris*, **251**, 442; 1960) described a female with eggs in the right oviduct (the left oviduct is non-functional) which appeared to confirm oviparity, as had been suggested by the apparent absence of any copulatory organ in the male, and by their earlier dissections of the urogenital systems and orifices of both sexes. Then a female was found with 19 large eggs in the oviduct, these apparently fully-developed eggs being 8.5–9.0 cm in diameter and weighing around 318 g. This appeared to clinch the matter, and Millot and Anthony's view was held by some as confirmed.

There were heretics, however. R. W. Griffith and K. S. Thomson (*Nature*, **242**, 617; 1973) pointed out that osmoregulatory demands would be too great to allow a shell-less egg of this size to develop in seawater. In addition, it seemed hard to believe that a fish of this size, with such a low fecundity could possibly have survived by simply laying tennis-ball sized eggs, even if they were hidden in a rock crevice or a nest. While the adult might have protected them from large predators, they would have been open to attack from the numerous crustacean inhabitants (particularly amphipods) of such a habitat. It would have seemed logical to assume that either *Latimeria* mouth-brooded its developing eggs (as do other marine fish which produce few large eggs), or more likely that the eggs developed internally.

Despite this, so deeply rooted was the notion that *Latimeria* was oviparous, that a recent authority (P. H. Greenwood, *A History of Fishes*, third ed., London, 1975) could write, "It must be assumed therefore that the fertilised eggs are physically unprotected; perhaps there is some form of parental care?"

The recent discovery by C. Lavett Smith and his co-workers thus puts an end to an era of muddled deduction. They report that five advanced young of lengths between 301 and 327 mm, and with moderately large yolk sacs (diameter 80–129 mm) were found in the specimen in New York. The young lie free in the oviduct and *Latimeria* is thus definitely ovoviviparous. Apart from their rather larger eyes and a more rounded head profile they resemble the adult closely. Fins and scales are well developed although they lack the fine teeth on the scales of the adult. Smith *et al.* point out that the female was captured in January (the same month in which females with ovulated eggs were taken) and they suggest from this that gestation may require more than a year.

Interestingly, the recent discovery that coelacanths are live-bearers was anticipated from the fossil record by nearly fifty years. D. M. S. Watson (*Proc. zool. Soc., London*, 453; 1927) reported the discovery of two small specimens of *Holophagus* (as *Undina*) within the body cavity of a larger specimen of this Jurassic fossil. It is pleasant that after this lapse of time Watson's observation on the fossil coelacanth has been confirmed by examination of the only living survivor of the group. □

The meiotic process

from a Correspondent

A Royal Society meeting for discussion on The Meiotic Process was held in London on December 10 and 11, 1975.

THE meeting explored fully the biological roles of meiosis: the halving of chromosome number in the formation of gametes and the synapsis and subsequent genetic recombination between homologous chromosomes. Opening the meeting, C. D. Darlington (University of Oxford) set the tone by pointing out that the chromosomes do not follow the laws of heredity, they make the laws. Almost every subsequent paper was concerned with one or other aspect of the movement, crossing over or disjunction of chromosomes in the two cell divisions which make up meiosis. The dominant theme was the study by electron microscopy of the synaptonemal complex (SC), an organelle of remarkably uniform dimensions and appearance in a wide range of eukaryotes, which mediates the pairing of homologues at the pachytene stage of meiosis.

Reconstructions of pachytene nuclei from serial sections show that the SCs extend along the whole length of the paired chromosomes (bivalents) and are usually attached at either end to the nuclear membrane. One of the major problems is to explain why bivalents do not become interlocked. S. W. Rasmussen (Carlsberg Laboratory, Copenhagen) showed that at an early stage in pairing in the silk worm, *Bombyx*, interlocking can in fact occur, but is in some way eliminated at a later stage. In this organism there is no crossing over or chiasmata formation in females and a progressive modification of the structure of the SC ensures that the chromosomes are held together until metaphase. D. von Wettstein (Carlsberg Laboratory, Copenhagen) showed that in normal

meiosis, where crossing over occurs, the SC is discarded after pachytene and can accumulate as polycomplex material dissociated from the chromosomes. A small part of the SC remains associated with the homologues at diplotene, probably at the points of crossing over. The importance of the recent discovery of nodules or nodes was emphasised by D. L. Lindsley (University of California, San Diego). These are discrete, densely stained bodies of unknown composition lying within or just outside the SC. Carpenter has obtained very suggestive evidence from *Drosophila* that the nodules in some way mediate or are associated with cross overs. Although a wealth of morphological information is available about the SC, so far almost no chemical work has been done. The major component is protein, probably with associated RNA, but it is completely unknown whether DNA enters the SC very occasionally at sites of crossing over, or at many sites, only a few of which generate cross overs.

Other approaches to the problem of pairing have been made with wheat and other cereals by R. Riley, R. B. Flavell and G. A. Dover (Plant Breeding Institute and Department of Genetics, Cambridge). In a wide range of cytological and physiological studies, the importance of the 5B and 5D chromosomes in controlling homologous and homeologous pairing has been established. It has been shown by temperature shift experiments and by colchicine treatment that events occur before the premeiotic S phase which are essential for normal chromosome pairing. The favoured hypothesis is that homologous chromosomes are pre-aligned at this stage and that a colchicine-sensitive fibrillar protein is involved. M. P. Maguire (University of Texas, Austin) also presented visible evidence for pre-aligned chromosomes in maize. M. D. Bennett (Plant Breeding Institute, Cambridge) showed that there is a linear relationship between DNA content and the duration of meiosis, which is independent of chromosome number. However, increases in ploidy within a species broke this rule and decreased the time of meiosis.

The importance of mutants in probing meiosis was best shown by the detailed studies of D. L. Lindsley and L. Sandler with *Drosophila*. A very large number of mutants are known which may specifically affect synapsis, crossing over or disjunction at the first or second division. Some are being exploited in further studies of the cross over 'nodule'. Many meiotic mutants also exist in yeast, and P. B. Moens (York University, Ontario) described two which are blocked in premeiotic DNA synthesis, but which nevertheless continue to proceed through meiosis

and form recombinants. One of the highlights of the meeting was the film by B. Nicklas (Duke University, North Carolina). He showed that by physically reorienting bivalents of grasshopper meiotic cells, the direction of movement of the chromosomes of metaphase I was determined by the direction in which the kinetochore (centromere) is pointing. Using the method of Telzer *et al.*, he has demonstrated *in vitro* that this may be related to the spontaneous association of tubulin with the kinetochores.

R. Holliday (National Institute for Medical Research, Mill Hill) discussed the likely mechanism of reciprocal and non-reciprocal recombination at the molecular level in relation to the processes of pairing and the formation of chiasmata. Cross overs between naked DNA molecules must be stabilised to form the chiasmata which hold the bivalents together, whereas non-reciprocal events may be transient. In an attempt to explain the ubiquitous phenomenon of interference between cross overs, he suggested that the SC may contain a limiting amount of a DNA-binding protein which is essential for their stabilisation. A major problem in our understanding of meiosis is the lack of biochemical information. The only systematic study has been made by H. Stern and Y. Hotta (University of California, San Diego) using *Lilium* and *Trillium*. In a review of the advances that have been made, Stern described the properties of the minor fractions of DNA synthesised at zygotene, which may be involved in pairing, and in pachytene, which may be due to repair synthesis during recombination. The latter is absent in the achiasmatic hybrid Black Beauty. Proteins which are synthesised at meiotic prophase and may play an essential role in recombination are a DNA-binding lipoprotein and an endonuclease.

Finally, D. Lewis (University College London) presented in one of the discussion periods an intriguing, although somewhat unrepeatable experiment. He described how in late Summer during the war, a V-1 flying bomb exploded at the John Innes Horticultural Institute, resulting two weeks later in out of season induction of meiosis and blossom in apple and particularly plum trees. It soon became apparent that the denudation of leaves was the main effect of the explosion. The result of this experiment, previously kept secret, indicated that the maturation of meocytes may be under the control of inhibitors, produced in this case by the leaves of the trees. This was certainly one of the most exciting experiments presented, and if published, the Materials and Methods should provide particularly interesting reading. □

review article

Scientific plans for deep sea drilling

C. A. Williams*

The Deep Sea Drilling Project is about to launch phase IV of its drilling operations, known as the International Phase of Ocean Drilling (IPOD). Plans for the first year of IPOD are well under way and a preliminary drilling schedule for the first four years has been drawn up. At this stage it is appropriate to inform the scientific community in general of these plans and to invite comment and suggestions. As in the past the scientific planning is carried out by the Joint Oceanographic Institutions for Deep Earth Sampling (JOIDES). This organisation, international from the outset, will through its various panels continue to provide scientific advice to DSDP for the IPOD programme as it has to the previous phases of drilling.

THE drilling project dates from the early 1960s when four major oceanographic institutions in the USA, Lamont-Doherty Geological Observatory of Columbia University, the Rosenstiel School of Marine and Atmospheric Science of the University of Miami, Scripps Institution of Oceanography of the University of California in San Diego, and Woods Hole Oceanographic Institution, formed JOIDES. An initial and successful drilling programme was carried out, under contract from the NSF to Lamont-Doherty, during the summer of 1965 on the Blake Plateau off Florida using the vessel Cardrill I. NSF next awarded a contract to the University of California for an 18-month programme, the Deep Sea Drilling Project (DSDP), in the Atlantic and Pacific Oceans. Phase I of this project, using the drilling vessel Glomar Challenger, began in August 1968. Since then the project has successfully continued through phases I-III, having completed phase III at the end of leg 44 in summer 1975. During this period approximately 400 holes have been drilled and ocean drilling pioneered in all major oceans.

The success of this project springs largely from the parallel programme of research and development into the technical aspects of drilling that has coexisted with the drilling operations, and which has provided both increased sophistication of methods and production in terms of material retrieved. Deeper holes can now be drilled with new improved drill bits, though perhaps the greatest advance is due to the development of the re-entry system, enabling a worn drill bit to be replaced and the hole re-entered for drilling to be continued. The next technical milestone will be the construction of a blow-out prevention system, which will open the way to a range of ocean margin sites presently inaccessible due to the possible encounter of high pressure fluids and gases within the sediments.

New emphasis during IPOD

IPOD will concentrate on drilling below the sedimentary cover of the ocean floors and intends to penetrate deeper into the ocean floor than ever before. There will also be

a greater concentration on fewer but deeper holes and an entire 56-d leg, or possibly longer, of Glomar Challenger's time will be spent on a single hole, penetrating to a depth of up to 2 km into the sea floor, IPOD I, lasting 4 yr, will continue to use the Glomar Challenger, but during IPOD II, when deep holes into marginal basins and deep-sea trenches are planned, it is anticipated that another drilling vessel, or platform, will be used.

Coupled with this re-emphasis, are changes in the organisation of JOIDES. After the initiation of JOIDES by the four US member institutions, the University of Washington became associated with the drilling project in 1968. During 1974-75 not only did the US membership increase to nine institutions with the addition of the Hawaii Institute of Geophysics, University of Rhode Island, Oregon State University and Texas A & M University, but it is now a formally recognised international organisation with member scientific institutions in the USSR, West Germany, France, Japan and the UK. Financial support for the programme continues to be mainly from the NSF, but with additional contributions from the member nations of IPOD/JOIDES.

The scientific approach of IPOD differs fundamentally from the previous drilling philosophies in that it is problem, rather than geographically, oriented. JOIDES advisory panels now include four problem panels concerned with the ocean crust, ocean margins (active and passive) and ocean palaeoenvironment.

Since effort will be concentrated on fewer sites, it is essential that the optimum locations be chosen; thus site surveying will also have a considerably more important role than it has during phases I-III. An IPOD Site Survey Management group, situated at Lamont-Doherty Geological Observatory, is designated to coordinate the survey programme and handle subcontracts for IPOD site surveys carried out by US institutions. The input of non-US data into this group remains on a voluntary basis and scientists who have relevant data are encouraged to submit this information for inclusion into the site data package for the chief scientists of that leg.

Atlantic drilling plans

Five of the first seven drilling legs to be spent in the North Atlantic will be devoted to the study of the oceanic crust. One of the main objectives is to establish a model for the

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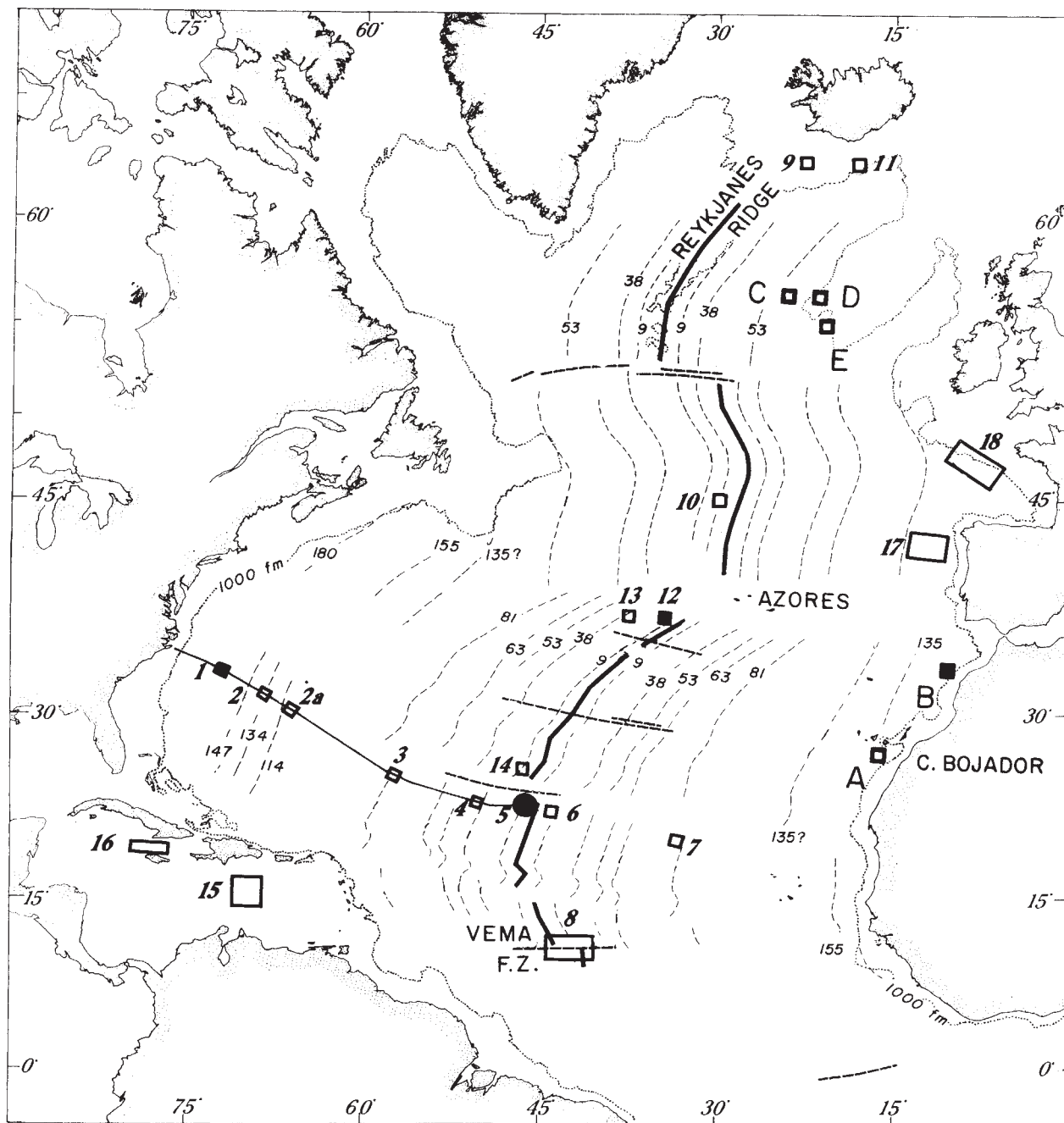


Fig. 1 Proposed IPOD Atlantic drilling sites. Open squares denote single bit sites, filled squares deep sites and filled circles very deep sites. The ages of the sea floor (Myr) are shown along magnetic lineations. The larger boxes indicate the region of several sites not yet precisely determined.

physical, geochemical, petrological, magnetic, thermal and other properties of the oceanic basement and to determine their range of variations, both with time (distance from the mid-ocean ridge crest) and location (along the length of the ridge crest).

The spatial and temporal problem will be tackled by way of two transects. An east-west transect along a 'flow' line (sites 1-7 in Fig. 1) will sample crust that has originated from the same spot on the ridge crest as determined from magnetic anomalies; in this way the variation with age away from the ridge crest will be investigated. The second transect, parallel to the ridge crest, will mainly be on magnetic anomaly 5 West (9-Myr-old sea floor) between latitudes 63°N on the Reykjanes Ridge and 12°N on the Vema Fracture Zone (sites 8-14). Local crustal variability will be investigated by means of shallow trial holes close to each site.

A test for the symmetry in crustal properties on either side of the Mid-Atlantic Ridge will be made by a comparison of samples at sites 3 and 5 with those from sites 7 and 6.

Sites are classified as being either single bit, that is, drilled to the limit of wear of one drill bit, which, depending on the hardness of the rocks encountered, could give a penetration of up to 500 m. Single re-entry sites which will penetrate deeper than 500 m and involve 6–10 d of drilling, and very deep sites involving multiple re-entry, penetration down to 2 km, and take 1–1½ legs of drilling. Atlantic site 5 (Fig. 1) is regarded as a prime site for a very deep hole and will be drilled early in the programme.

The sediments at all holes will be continuously cored and it is hoped that a logging programme can be implemented for some of the holes. Site 10 will be near 45°N. Site 14 is in the trans-Atlantic geotraverse corridor near 26°N, both

are areas well investigated by previous work and requiring little or no additional site surveying. Five holes are planned at Site 8 in the Vema Fracture Zone area. These will be to investigate the nature of the fracture zone and will also extend the longitudinal transect southwards to 12°N.

The original intent of the longitudinal transect was to sample the crust at shallow sites more or less evenly spaced along the length of the Mid-Atlantic Ridge on magnetic anomaly lineation 5 west. Site 9 will be on magnetic anomaly 5 east and site 11 on the same 'flow' line but on older oceanic crust at anomaly 13 (38 Myr), to investigate the Iceland geochemical anomaly. This represents a trend away from the initial purist approach of avoiding as far as possible any known geochemical anomaly. The change in policy represents both the scientist's inherent propensity to investigate anomalies, or recognition that simple situations only exist where investigation has not yet proved otherwise. As our knowledge of the ocean floor grows, it may become apparent that it is impossible to avoid anomalies. Site 13 will be on anomaly 13 west, along the corresponding flow line west of the FAMOUS area, where, as a result of the very considerable Franco-American effort to study a small area of the Mid-Atlantic ridge crest in great detail, perhaps more is known of the ocean floor than in any other area. Site 12, which will be a deep site near DSDP site 334, and Site 13 will thus form a separate east-west mini-transect in the same way as sites 9 and 11.

Of the first few legs to be spent in the North Atlantic, two of these will concern themselves with the problems of the continental margins. At this stage, four specific areas have been designated: Off north-west Africa, sites A and B, Galicia Bank (area 17), the northern margin of the Bay of Biscay (area 18) and Rockall Bank sites C, D and E (Fig. 1). The first of these sites to be drilled will be site A off Cape Bojador, and a re-entry site in the Morocco Basin. Here it is hoped to sample pre-Oxfordian sediments in the North Atlantic for the first time. Both these holes will also investigate the nature of the sediments relating to the history of this margin during the opening of the Atlantic Ocean approximately 180 Myr ago.

Three sites will be located on Galicia Bank, the submerged continental block which enigmatically does not fit into the reconstruction of the Atlantic continents. The age of the oldest sediments overlying the oceanic crust will be sampled to the west of the bank, and then at a site on one of the faulted blocks on the south or western slope of the Bank, information will be sought on the age and processes involved during the initial rifting apart of the continents. A site on the top of the carbonate platform will investigate the stratigraphy and subsidence history of the platform itself.

Another designated area of interest is the northern continental margin of Biscay, where at three single-bit sites the history of subsidence and sedimentation of this region will be investigated in the light of the history of opening of the Atlantic and Bay of Biscay. Three sites (C, D and E in Fig. 1) are also proposed near the south-west end of Rockall Plateau where the objective is to investigate the history of subsidence of the margin relative to the three different phases of continental separation which have occurred peripherally to Rockall Bank, during the Lower Cretaceous(?), at 80 and 60 Myr.

Additionally, a leg may be devoted to the Norwegian Sea where both crustal and margin problems will be investigated. This programme is still in its initial planning stages, and the proposals for this area will shortly be reviewed by the JOIDES ocean crust and safety panels.

Palaeoenvironmental aims

The Ocean Palaeoenvironment Panel is interested, through the study of faunas and sediments, in developing a model

for the opening and subsequent evolutionary history of the ocean basin from the point of view of their palaeoclimate, palaeocurrents and so on. The panel also has more specific aims, including a study of the early history specifically of the Atlantic, and to investigate the Cretaceous-Palaeogene boundary as an example of a palaeoecological crisis. Many of their other interests in global ocean circulation, the evolution of plankton communities through time, and so on, require a more extensive global coverage of data than do those of the other panels, and the Palaeoenvironment Panel would like to drill in the Indian Ocean, Antarctic and the South Atlantic as well as in the North Atlantic and North Pacific. There are, however, already more proposals than can be fitted into the programme and even with the recent extension of IPOD I from a 3-yr into a 4-yr programme, it will not be possible to drill in the Indian Ocean or the Antarctic during IPOD I. Two palaeoenvironment legs are planned, however, in the South Atlantic where sections across the Angola and Cape Basins will further investigate the effect of the Walvis Ridge on the early history of sedimentation in the Atlantic.

The development of early plankton communities can be investigated in the northern Pacific, where a hemispherical ocean with an uncomplicated gyral current system is required. Eighteen holes are proposed across the northern Pacific for this programme. The Cretaceous-Palaeogene boundary can be investigated near the Sierra Leone Rise in the Atlantic and also at sites near Rockall Bank, in the Caribbean and in the NW and equatorial Pacific, which will tie in with the other parts of the programme. Volcanic episodicity can also be studied from ash layers in the sediments at sites in the Sea of Okhotsk, the Japan Sea, off Central Chile and also at ocean crust sites near Hawaii and Iceland.

Pacific drilling plans

Drilling plans for the Pacific are still in outline (see Fig. 2), but the programme will be planned in many respects to parallel the Atlantic work, to compare the slow spreading (half rate of 1–2 cm yr⁻¹) Atlantic with the faster spreading (6–10 cm yr⁻¹) Pacific. It is not feasible to follow a single flow line across the Pacific as in the Atlantic, and hence the major traverses are split between several flow lines. Two, possibly alternative sites (1 and 3 in Fig. 2) have been chosen to sample very young ocean floor. These candidate sites in the Gulf of California and on the Juan de Fuca Ridge, are both where sufficient sediments to support, or 'spud in', the drill bit occur at the ridge crest. Such sites should provide information on hydrothermal circulation within the crest which may only occur near the ridge crest, on thermal gradients and should also provide unweathered samples for geochemical analysis. The presence of sediments at the ridge crest probably causes an intermixing of lava and sediment and implies that the basement here could be anomalous; however, the value of obtaining very young samples is considered to outweigh this factor.

The fastest Pacific seafloor spreading area, at 20°S, is remote, has a confused magnetic pattern, a thin sediment cover and was rejected in favour of the Siqueiros Fracture Zone at approximately 8°N on the eastern north Pacific (site 4). This relatively fast-spreading transect is planned with sites on 2- and 5-Myr-old crust in sediment ponds existing within 10–20 km of the ridge crest and where seismic refraction data indicate a low velocity zone (possibly the magma chamber?) centred beneath the ridge crest. The spreading rate here was approximately 5 cm yr⁻¹ over the past 10 Myr, three times that of the Atlantic and adequate to compare the evolution of fast and slow spreading ridges.

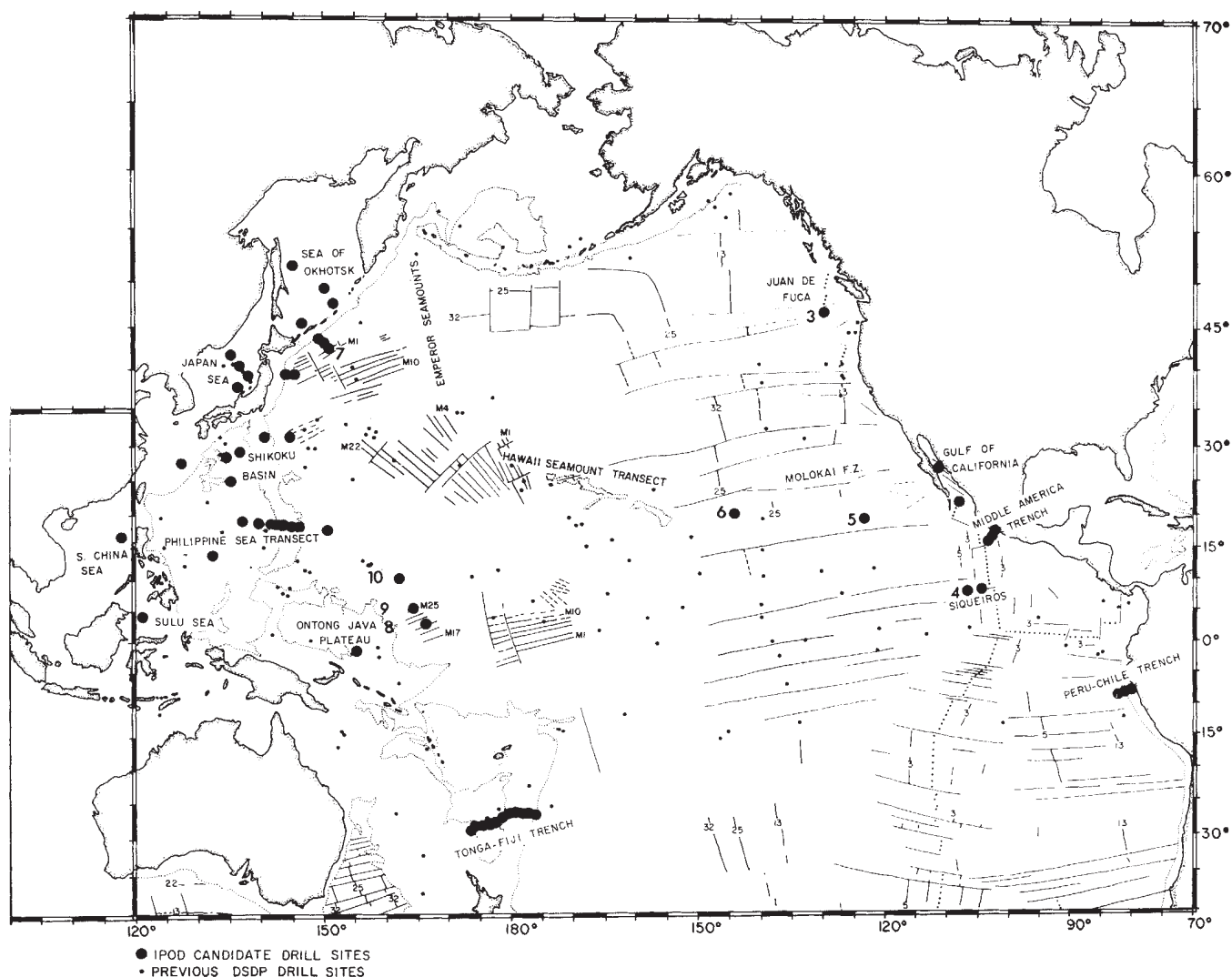


Fig. 2 Proposed IPOD Pacific drill sites. The larger circles denote the proposed IPOD sites and the smaller circles show previously drilled DSDP sites. The multi-site traverses are indicated diagrammatically since the scale of the map does not allow otherwise.

This will be one of the high priority areas for very deep penetration.

Pacific sites 5 and 6 will be shallow holes on anomalies 10 (33 Myr) and 32 (76 Myr) respectively and lie on a flow line south of the Molokai fracture zone which will tie in with the Gulf of California sites.

The oldest known Pacific sea floor and the Jurassic quiet magnetic zone will be sampled at sites 7–10 in the western Pacific. Site 7 will lie on magnetic anomaly M1 (116-Myr-old sea floor) south of the Kurile trench and will date this important magnetic anomaly and also tie in with a traverse from the Sea of Okhotsk across the Kurile trench chosen by the Active Margin Panel. Traverses across the Sea of Okhotsk, the Shikoku Basin and south Philippine Sea are designed to investigate the formation of back-arc and marginal basins. The Philippine traverse comprises thirteen proposed sites with the intention of sampling the floor of the South China Sea, the W. Philippine Sea, the Palau-Kyushu Ridge, the east and west Parece-Vela Basins, the Mariana Islands, trench gap and trench. The results from these sites may allow for clarification between the two current conflicting hypotheses for the origin of the floors of marginal basin areas; that they are areas of entrapped old Pacific sea floor, or alternatively crust formed by sea-floor spreading within the marginal basins themselves.

A transect across the Japan Sea is planned with a series of up to 8 holes to investigate the nature of the crust in that area. All but two of these, and two of the South Philippine transect sites, demand penetration beyond the present capabilities of *Glomar Challenger* with a 6.7-km long drill string and will be candidate sites for the second phase of IPOD drilling when an 11.0-km drill string is planned. Other active margin traverses are also planned across the Tonga-Fiji trench, the Middle America Trench and the Peru-Chile trench. In due course these will be categorised into priority and contingency sites as the Pacific drilling plan evolves.

The IPOD I programme is scheduled to spend the first eight legs in the Atlantic and Caribbean, the following 12 legs in the Pacific and will return at the end of IPOD I for five final legs in the Atlantic. Clearly the candidate drill sites are already too numerous for all of them to be drilled and the task of the JOIDES advisory panels will be to pare down this number into a workable drilling plan with both established priority and contingency sites.

The JOIDES organisation is keen to hear from the scientific community at large and suggestions are welcome as to precise drilling locations, scientific staffing of the *Glomar Challenger* for specific legs, and the organisation and allocation of effort with respect to shore-based study of core material, together with relevant existing site survey data.

articles

Observations of solar pulsations

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We have modified our solar magnetograph to measure velocities at the solar surface, rather than magnetic fields. Using this apparatus, we have observed fluctuations of period 2 h 40 min, which are remarkably stable. The interpretation of this phenomenon seems to cause much theoretical difficulty.

WE have reported previously^{1,2} on the successful measurement of the Sun's magnetic field using a solar magnetograph and the Crimean Solar Tower Telescope. The magnetograph consists of an electro-optical modulator to pick out the circularly polarised Zeeman components of magnetically sensitive lines, a grating and photomultipliers set on the wings of the lines. We thus achieve sensitivity, but, with the aid of an automatic Doppler compensator, avoid the perturbations of varying relative velocities between the Earth and Sun, turbulence in the atmosphere and apparatus noise. By the use of some additional apparatus we have now investigated periodic fluctuations in the Sun's surface.

Method and apparatus

To avoid unnecessary complications, we work with a magnetically insensitive line ($\lambda = 5,123.7 \text{ \AA}$). A circular polariser was placed between the telescope and the entrance slit of the spectrograph, of such a diameter to polarise light from the central portion of the Sun only. Light from the solar rim will always reach the photomultipliers (it is initially unpolarised), but light from the central disk will get through only when the magnetograph polariser (modulated at 760 Hz) is set for it. The difference between the outputs of the two photomultipliers provides the magnetograph signal, and the different signals for the two polarisations indicates whether or not light from the centre is shifted relative to that from the rim. The Doppler shift can be converted into a line-of-sight velocity.

To avoid effects arising from the great rotational velocity of the equatorial parts of the Sun, we looked at light from a pole-pole strip across the Sun only. We calibrated the instrument with a line of known intensity and wavelength to relate a known wavelength shift to the measured signal. The interpretation of a positive experimental signal depends on the geometry of the apparatus (the ratio of the area of the polar image to that of the central image) and the mean relative emissivities of the polar regions and central ones.

With our particular geometry, we have derived the following formula for the experimental signal $\delta_{||}$ in terms of the wavelength shift $\Delta\lambda_v$, assuming a triangular line profile

$$\frac{\delta_{||}}{\delta_{||}(O)} = \frac{1}{2 + \beta} \frac{\Delta\lambda_v}{\Delta\lambda_c} \quad (1)$$

Here $\Delta\lambda_c$ is the wavelength shift we produced artificially for the

calibration signal $\delta_{||}(O)$, with the aid of the plane-parallel glass plate of the velocity compensator (which is 'off' for calibrations, and 'on' for recording $\Delta\lambda_v$), and β is the ratio of the intensity at the central part of the solar image to that at the rim, $\beta = I_c/I_r \approx \frac{1}{3}$ in our case (intensities measured by photomultipliers). The calibration is made for a standard shift $\Delta\lambda_c = \pm 0.031 \text{ \AA}$ of the $5,123.7 \text{ \AA}$ line, which corresponds to velocity of $\pm 1,815 \text{ m s}^{-1}$, so that $\delta_{||}$ equals $\delta_{||}(O)$ when $\Delta\lambda_v = 2.34 \times \Delta\lambda_c$ —for a speed of $4,238 \text{ m s}^{-1}$.

Accuracy of experiment

The accuracy of the method is limited by turbulence in the spectrograph and electronic noise, and can be determined from the accuracy of magnetic field measurements³: where mean field strengths of 1 gauss were measured, corresponding to $\approx 2.2 \text{ m s}^{-1}$. By increasing the data accumulation time to 15 min, we can achieve $\approx 1.0 \text{ m s}^{-1}$, corresponding to a Doppler shift $\Delta\lambda_v \approx 10^{-5} \text{ \AA}$.

A special auxiliary guiding system ensured angular accuracy of $\pm 0.1'' \times 0.2''$ in positioning the beams. There were also effects varying regularly during the whole day connected with slowly changing imbalances in both the electronic channels of the magnetograph, or of photoguiding system, but this slow run of the signal was easily eliminated (especially when we compared the records of two independent magnetographs in our double-channel magnetographic system⁴). In all, 122 h of data (from 21 d) were obtained (August–October 1974 and March 1975).

Analysis

Analysis showed at once the existence of small fluctuations of signal $\delta_{||}$ with mean characteristic times grouping around 2 h 40 min. The extrema of $\delta_{||}$ occurred independently of the local time, and did not correlate with the start of observations (which lasted from 3 to 10 h continuously, depending on weather). This shows definitely that the effect is connected neither with periodic error in the drive of the coelostat nor with the possible heating of optics and misalignment between the main and the guiding system. Moreover, at the beginning of the work we made records of the signal $\delta_{||}$ in two lines, the solar line ($\lambda 5,123.7 \text{ \AA}$) and the telluric line ($\lambda 5,901.5 \text{ \AA}$), simultaneously using both channels of the magnetograph to check if the effect was real or instrumental. One of these records, presented in Fig. 1, shows clearly that fluctuations arose from the Sun itself. We afterwards used only the solar line $\lambda 5,123.7 \text{ \AA}$.

To find the approximate periods of the fluctuation, we overlapped all fluctuations for the first nine days in August 1974 on the same graph to get the best fit (coincidence of maxima and minima) between them (see Fig. 2). This shows an almost sinusoidal wave of period $\sim 2 \text{ h } 40 \pm 5 \text{ min}$. We then applied superposed epoch analysis, and folded all data from

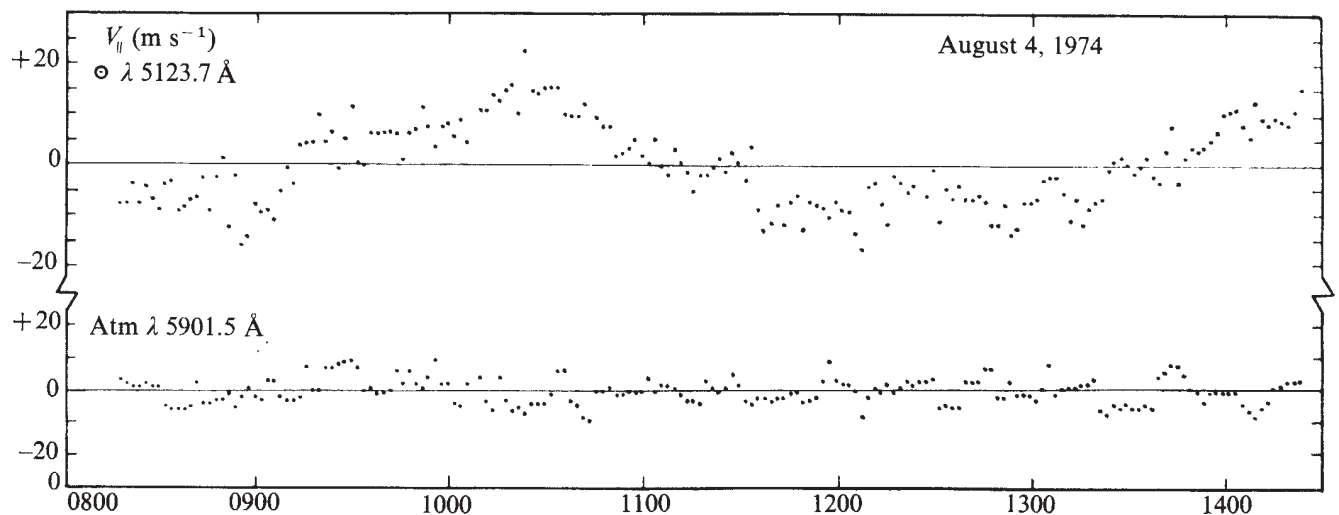


Fig. 1 Measurements of Doppler shift $\Delta\lambda_v$ made on August 4, 1974, simultaneously on the solar line ($\lambda = 5123.7$, $\text{\AA}$, top) and telluric line ($\lambda = 5901.5$, $\text{\AA}$, bottom). The horizontal axis represents the local time.

August–September, with this approximate period 2 h 30 min–2 h 50 min (with the steps of 0.5 and 1 min) looking for the wave with minimum σ^2 deviations. The best fit was for 2 h 40 $\pm$ 0.5 min (Fig. 3). The mean amplitude of radial velocity $v_{||}$ is $\approx \pm 2$ m s $^{-1}$ so that the amplitude of pulsations is ≈ 10 km.

The observations made in October 1974 and March 1975 also showed the same periodicity occurred almost in phase with the data from August–September 1974 (stability over $\sim 1,900$ periods). Therefore the period can be determined in fact with much higher accuracy than quoted above (± 0.5 min).

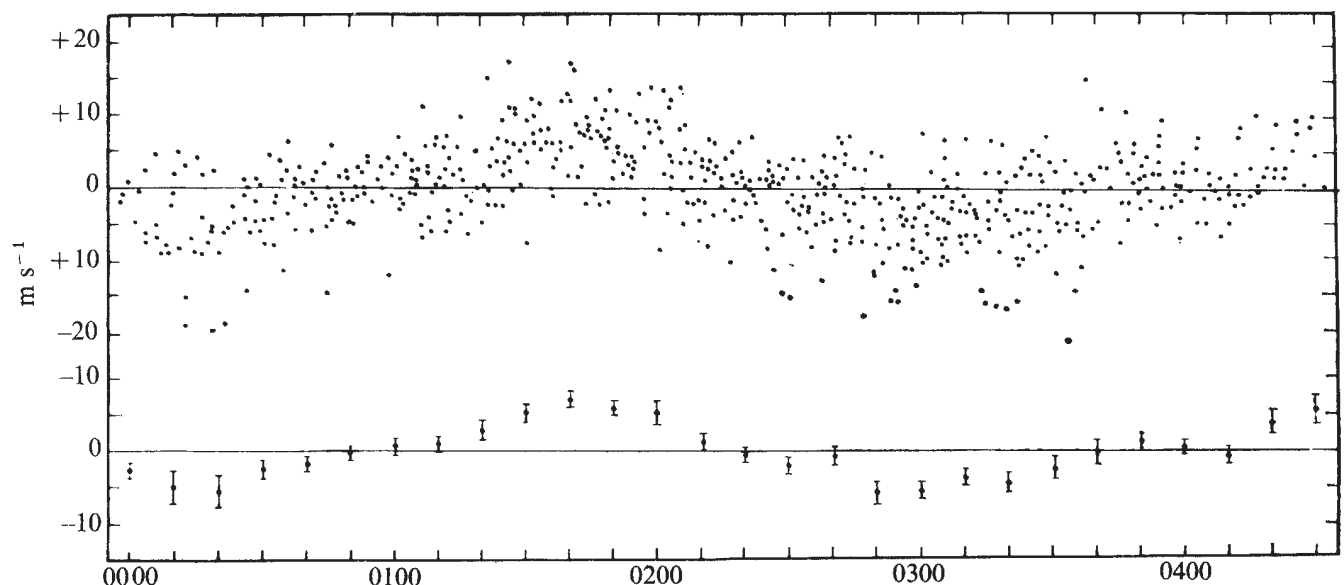
Interpretation

It is tempting to speculate about the results obtained. The systematic appearance of stationary periodic fluctuations in radial velocities of large (comparable with the radius of the disk) portions of the solar surface points definitely to pulsations of the Sun as a whole, and not to the rather stochastic processes of the ascent and descent of gigantic supergranules. The period 2 h 40 min is too long to be ascribed to the envelope of the Sun (the region where gravity acceleration $g \approx$ constant), where the period would be no longer than 40–60 min. It also

cannot be ascribed to a set of standing oscillations on the solar surface⁴, because the effect does not depend on the phase of solar rotation. It cannot be a travelling wave around the Sun because the velocity of such a wave would be too high, ≈ 140 km s $^{-1}$.

The simplest interpretation is that we observed purely radial pulsations. The most striking fact is that the observed period 2 h 40 min is almost precisely the same as that following from the simple formula of Ritter for radial pulsations $\sigma^2 = (3\gamma - 4)g/R_\odot$ giving $P = 2\pi/\sigma = 2$ h 47 min for $\gamma = 5/3$, almost the value if the Sun were to be an homogeneous sphere. (It should also be noted here that for more inhomogeneous models, for example when $\rho \sim r^{-2}$ or on Roche's model, we would get much shorter periods.) The question can be considered more accurately using the results of Chandrasekhar and Lebowitz⁵, or those of Cox *et al.*⁶ for the oscillations of polytropic gaseous spheres. It is easy to find from these results the dependence of $\sigma^2/4\pi G\rho_m\lambda$ on the increase in density $\lambda = \rho_c/\rho_m$ towards the centre of the Sun, which shows that periods decrease with increase of λ and of polytropic index n (as well as with $3\gamma - 4$) for all different modes of oscillations (pulsational, transverse

Fig. 2 The best fit for the fluctuations from visual inspection of the records obtained during first 9 d of observations. Each point is the 5-min integrated signal of radial velocity (top). Averaging this gives the sine-like curve shown below.



shear, and toroidal, modes). For all these, the periods seem to be too short to be compatible with observations unless the polytropic index is not higher than $n \approx 0.5$. If, for a moment, we assume such a value of n , it would lead us to $\lambda \approx 1.5$, $\rho_c \approx 2$ and $T_c \approx 6.5 \times 10^6$ K, and, at the mean molecular weight $\mu = 0.6$ ($X = 0.73$, $Y = 0.26$, $Z = 0.014$)⁷, we would get such a low rate of energy generation ϵ from proton-proton reactions (see Reeves⁸, formula (3.6)) that the luminosity

$$L = \int_0^{R_\odot} \epsilon(p, p) 4\pi r^2 \rho dr$$

would be $\approx 2.5 \times 10^{20}$ erg s⁻¹, $\sim 10^4$ times smaller than the observed value.

Possible explanation

We have investigated two possible solutions to this dilemma. The first alternative is that nuclear, and, in particular, (p,p) reactions are not responsible for energy generation in the Sun. Such a conclusion, although rather extravagant, is quite consistent with the observed absence of appreciable neutrino flux from the Sun⁹, and with the observed abundance of Li and Be in the solar atmosphere. The second alternative pointed out to us by D. Gough, personal communication) is to adopt the current model of solar structure with (p,p) reactions and assume that we really observe not pure radial pulsations but some gravity g mode of quadrupole oscillation. (The possibility of deformations of the type was also pointed out to us by Schatzman at our preliminary report on this work at the colloquium at Meudon observatory, May 21, 1975.) g modes ($l = 2$) can yield long period oscillations¹⁰, and according to Gough's calculations (private communication) the period of the g_{11} mode is in perfect agreement with our observed period 2 h 40 min. It seems strange, however, that this high harmonic is dominant.

We should keep in mind that our present method does not allow us to distinguish pure radial pulsations from quadrupole oscillations. Neither does it allow us to observe dipole ($l = 1$) oscillations at all. Our observations do, however, give some hint of small amplitude oscillations of a shorter period overlapped on the main 2 h 40 min oscillation. Spectral analysis of corresponding data will be given elsewhere.

We are inclined to believe that the problem arising here is much the same as in the case of Cepheids discussed long ago

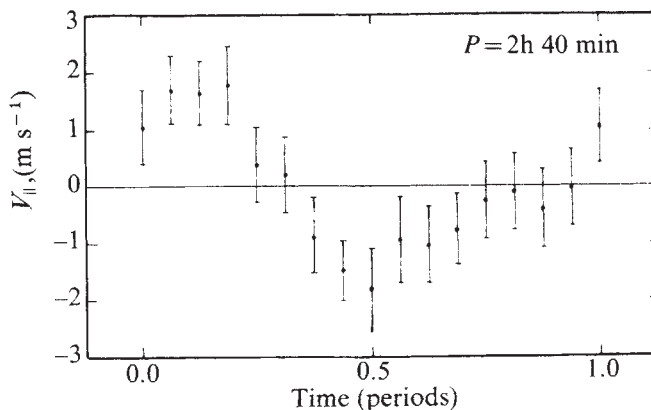


Fig. 3 Result of the superposed epoch analysis with 2 h 40 min period. Error bars represent r.m.s. deviations of individual measurements for each 10-min block of the data.

by Eddington¹¹ and Rosseland¹², where the best agreement with observations can be reached for almost homogeneous spheres at $\gamma = 5/3$. We agree with Rosseland's conclusion that 'a thorough discussion of the problem on a new basis seems to be called for.'

We thank Dr D. Gough for helpful discussion of the paper before publication.

Note added in proof: Preliminary results of observations for 16 d in 1975 show the same periodicity in the mean magnetic field of the Sun, with an amplitude of 0.01 gauss.

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Towards a heliological inverse problem

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Theoretical periods of normal modes of vibration of the Sun are compared with the observed periods of oscillation of the solar surface. It is inferred from the comparison that it may soon be possible to use solar oscillations to measure aspects of the internal structure of the Sun.

THE observational data that have been available for comparison with theoretical solar models are insufficient to enable stellar astrophysicists to ascertain the internal structure of the Sun. These data are just gross properties, such as the age, mass, radius, photon luminosity and neutrino luminosity, together

with details of the optical spectrum which are relevant only to the solar atmosphere. But recently there have been accurate measurements made of the frequencies of what are presumably normal modes of vibration of the Sun¹⁻³. These dynamical data provide us with a new kind of information with which to probe the solar interior, and might lead to quite sensitive measures of certain aspects of the Sun's structure.

Models of the Sun

Let us consider first how standard solar models are constructed. They are spherically symmetrical, and in hydrostatic and thermal balance. The assumption that the rotation rate in the

interior is comparable with that observed at the surface is usually made, which leads to negligible centrifugal forces and implies that spherical symmetry is a good approximation. This is consistent with the observed near circular shape of the solar image. It also leads to very low estimates of rotationally induced circulation currents. Thus, since solar models have convectively stable cores after the first few hundred Myr of their evolution on the main sequence, the products of thermonuclear reactions after that time are assumed not to have been transported away from the place where they were produced.

Models of stars of solar mass constructed from these assumptions predict a relationship between the photon luminosity L and time. This can easily be made consistent with the present solar luminosity $L_{\odot}$ by adjusting the initial composition within the quite broad limits set by observation. Indeed, for any plausible initial abundance Z of heavy elements (usually assumed uniform) an initial hydrogen abundance X can always be found that reproduces $L_{\odot}$. The solar radius is not predicted because our ability to model the convection zone in the outer part of the star is inadequate. Instead convection theories are calibrated by adjusting solar models to have the correct radius at the appropriate age.

Thus, before the results of the attempt by Davis and his collaborators^{4,5} to detect solar neutrinos were available, there was at least a single infinity of acceptable solar models, with varying Z , from which to choose. One of the roles of Davis' experiment was to help make the choice. It was not anticipated that the measured upper bound to the neutrino luminosity L_{ν} would be a factor of ~ 10 lower than the lowest theoretical predictions. Subsequently, models with low values of L_{ν} were constructed⁶⁻¹³, though they did not satisfy all of the assumptions mentioned above. No doubt many more will emerge in the future. Some can be eliminated on theoretical grounds, such as those with hydrogen shells¹² or cores¹³ which are Rayleigh-Taylor unstable, but the rest must be tested against new observations.

Solar oscillations

An hypothesis that has received much attention in recent years is that the Sun has a rapidly rotating core. This has been pursued partly to produce models with low L_{ν} , but principally to attempt to refute general relativity¹⁴. Careful observations of the oblateness of the quiet Sun by Hill and Stebbins¹⁵ now makes the hypothesis unlikely. But these and other similar observations^{1,16} have revealed that the apparent diameter of the solar image is oscillating, with a discrete spectrum of periods $\lesssim 1$ h, and in the range of the low order p modes (acoustic modes). Severny, Kotov and Tsap² (in this issue of *Nature*) report observations of the difference between line of sight velocities at the poles and disk centre. This too is oscillatory, with a dominant period of 2 h 40 min, and is probably associated with a g mode (gravity wave). A spectrum of oscillations in a mean line-of-sight velocity has also been obtained recently by Brookes, Isaak and van der Raay³ (also in this issue of *Nature*), who compared solar absorption lines with corresponding lines produced in the laboratory. The largest period observed is 2.65 ± 0.04 h, in agreement with Severny *et al.* Fossat and Ricort³⁶ have recently reported large scale oscillations with a period of about 10 min.

Oscillations of the solar atmosphere have been observed before: the five-minute oscillations have been studied for more than a decade¹⁷, but these are probably a superposition of only those normal modes that have significant amplitudes in the extreme outer layers of the Sun (ref. 18, and H. Ando and Y. Osaki, unpublished), and so provide no direct information about the interior. Some 'long period oscillations' with periods of 40-50 min have also been detected¹⁹⁻²², but their periods were not accurately determined.

All the oscillations observed have too low an amplitude to influence the structure of the solar interior significantly, but their presence provides a new rich class of diagnostic data with which to compare the properties of theoretical models.

Oscillations and solar structure

To what extent can the structure of the Sun be inferred from these comparisons? As a first step towards answering this question we have computed the low frequency ends of three spectra of p modes and the high frequency end of one spectrum of g modes. The p modes chosen have $l = 0, 2$ and 4 and the g modes have $l = 2$, because these are the modes most likely to have been detected with the present techniques. The basic solar model was computed in the standard way with the program described by Eggleton²³. With $Z = 0.02$ and $X = 0.735$ at zero age, and a mixing length of 1.10 pressure scale heights in Böhm-Vitense's²⁴ formulation of the mixing length theory, the model evolved to the present luminosity and radius in 4.7×10^9 yr. The linearised oscillations were computed in adiabatic approximation in the manner described in ref. 25; non-adiabatic processes have a negligible influence on the frequencies of those modes with few nodes in the radial direction. The results are presented in Table 1, together with the frequencies of the peaks in the spectra reported by Hill, Stebbins and Brown¹⁶. To gain some idea of the sensitivity of the computed frequencies to variations in the basic solar model, the calculation was repeated with a heavy element abundance $Z = 0.04$. This required choosing $X = 0.68$ at zero age and a mixing length of 1.325 pressure scale heights. The frequencies of a few of the modes are presented in Table 1 in parentheses. The frequencies in square brackets were obtained by changing the distribution of mesh points, and so indicate a lower bound to truncation errors.

Several features of Table 1 are noteworthy. First, it is clear that all but perhaps the lowest of the observed frequencies are approximately reproduced by the theory. This is evidence that the oscillations are not spurious and that most of them are indeed p modes. Not all the modes computed have been detected, however, though there is some evidence from the observed power spectrum displayed by Hill, Stebbins and Brown¹⁶ for the existence of modes not listed in Table 1. For example, the p_4 ($l = 0$) and p_3 ($l = 2$) modes have frequencies near 0.63 mHz which is the position of a prominent shoulder in the power spectrum. Second, the frequencies of the radial ($l = 0$) modes and the quadrupole ($l = 2$) modes are very similar. Third, the effect on the p mode frequencies of changing Z is no greater than the observational uncertainties and the truncation errors in the calculations. The influence of composition changes on the g mode frequencies is somewhat greater, presumably because g modes are more concentrated towards the centre of the Sun, where the structure is more sensitive to the opacity. Indeed there is no g mode with $l = 2$ (and none with $l = 4$) with a period within the quoted errors of the observed period of 2 h 40 $\pm$ 1 min when $Z = 0.02$, but there is when $Z = 0.04$. This cannot be considered as evidence for the higher Z , however, partly because the calculations are not sufficiently reliable, and partly because the identification of the mode may be incorrect: the oscillation may be a g_{10} ($l = 2$) mode with $Z \lesssim 0.02$. (It is also evident that a very drastic change in the solar model would be necessary to enable the 2 h 40 min oscillation to be interpreted as the fundamental radial mode, as Severny *et al.*² and Brookes *et al.*³ suggest. Indeed it is unlikely that any such model can be found that can generate the observed photon luminosity by thermonuclear reactions.)

Future work

Although we are not yet in a position to draw definitive conclusions concerning the internal structure of the Sun from these observations, the results of this investigation do suggest that useful diagnostics from frequencies of oscillation will be available in the near future. To achieve this, greater accuracy must be attained from both the observations and the theory. Theorists have usually not considered it worthwhile to take the trouble to achieve high accuracy in the numerical integrations of the equations of stellar evolution, partly because our knowledge of opacities and nuclear reaction rates is uncertain and because

Table 1 Periods of solar oscillations (min)

1973	1975	$l = 0$		$l = 2$		$l = 4$		$l = 2$	
		Mode	Period	Mode	Period	Mode	Period	Mode	Period
		p_1	62.7 (63.9)					g_1	55.1
52	47.9			f	46.0				
		p_2	43.8	p_1	42.2 (42.3)	f	39.6	g_2	61.5
33		p_3	32.6 (31.8)			p_1	38.0	g_3	70.9
	30.3	p_4	26.0	p_2	34.3	p_2	29.3	g_4	81.8
23.8		p_5	21.0 [21.5]	p_3	26.7	p_3	23.4		
	21.0			p_4	21.5			g_5	93.0
		p_6	18.2	p_5	17.9	p_4	19.5	g_6	105
16.7	17.1	p_7	15.8	p_6	16.0	p_5	16.8	g_7	117
	14.6	p_8	[15.4] 14.0	p_7	14.1	p_6	14.6	g_8	130
13.3		p_9	12.5	p_8	12.6	p_7	13.0	g_9	142
11.9	11.8	p_{10}	11.3	p_9	11.4	p_8	11.7	g_{10}	154 (147)
10.4	10.5	p_{11}	10.4	p_{10}	10.4			g_{11}	167 (159)
		p_{12}	9.55					g_{12}	180 (171)
9.2	8.8	p_{13}	8.84 [9.12]						
		p_{14}	8.52						
	7.9	p_{15}	7.99						
7.6		p_{16}	7.53						
7.0	7.2	p_{17}	7.12						
		p_{18}	6.75						
		p_{19}	6.41						
		p_{20}	6.10						

The periods headed 1973 and 1975 are from the observations reported by Hill, Stebbins and Brown¹⁶, and are accurate to $\sim 5\%$. Those periods not reported in the publication were kindly supplied by Professor Hill. Periods in the other columns were obtained theoretically using a solar model with $Z = 0.02$, except those in parentheses which are periods of oscillation of a model with $Z = 0.04$. The periods in square brackets were obtained by repeating the computation with $Z = 0.02$ using a different distribution of mesh points. The classification of the modes, radial and non-radial, is according to the scheme proposed by Scuflaire³⁴ and Osaki³⁵.

we cannot model thermal convection and other macroscopic motions reliably, and partly because there have been no observations that would distinguish between subtle differences in the models. But now the time has come to be a little more careful. Potential second-order accuracy difference schemes in some evolution programmes are spoiled, for example, by linearly interpolating opacity tables; this can quite easily be avoided. Accurate frequencies can be achieved by using variational principles on computed eigenfunctions. Also, more accurate observations are imminent. We do not know for how long a particular normal mode maintains an observable amplitude, but if it is many years, frequent observations over a long interval of time will yield very accurate periods. The observations by Severny *et al.*² and Brookes *et al.*³ are a first step in this direction. Longer observing programmes are planned by Brown, Hill and Stebbins (personal communication) which may fix the p mode frequencies to within $\frac{1}{4}\%$, and make g mode periods measurable by their technique.

Prospects

The prospect of such information is exciting, for it opens the possibility of a limited inverse problem of much the same kind as geophysicists pose to probe the internal structure of the Earth with seismic waves^{26,27}. Because a spectrum contains so much information one can attempt to measure functions of radius r rather than just integral quantities such as luminosity. For example, given a certain set of assumptions such as hydrostatic support, thermal balance, an equation of state, opacities and nuclear reaction rates, one can seek that Z and that function $X(r)$ that reproduces the observed spectra. If they do not

exist the assumptions must then be questioned: the answers may give a clue as to why standard solar models have been unable to yield neutrino fluxes consistent with Davis' observations.

To perform such an inversion, the modes exhibiting the observed frequencies must be correctly identified. It is apparent from Table 1 that it would be extremely difficult, if not impossible, to distinguish between p modes with $l = 0$ and $l = 2$ by frequency measurements alone, because the differences between their frequencies are less than variations that are produced by plausible changes in the composition of solar models. Instead, l must be measured directly, by observing the dependence of the amplitude and phase of the oscillations with position on the solar image. Hill and his collaborators plan to measure latitude dependence in the near future. This will yield several spectra, which in principle could be used to estimate simultaneously several functions such as $X(r)$. With enough spectra, a detailed model of the present Sun might be constructed without recourse to assumptions about the solar history. Of course since only finite portions of the infinite spectra can be measured, only limited resolution can be achieved, but with reasonable assumptions about smoothness one might hope to achieve considerably more reliable models than we have at present.

It is necessary to ask how many different oscillation spectra will be required to achieve this. The answer is not yet known, though Backus and Gilbert's²⁷ speculations on the geophysical inverse problem suggest that the number is somewhat greater (perhaps by almost a factor 2) than the number of functions to be measured. In any case, the reliability of the inferences increases with the number of spectra available.

It would thus be useful to add to the spectra that are currently being measured, the modes with $l = 1$. These modes are antisymmetrical about the Equator (assuming the axis of the mode coincides with the axis of rotation). The line-of-sight velocity varies with latitude λ as $\sin 2\lambda$ and so vanishes at pole and Equator, but the technique described by Severny *et al.*² could presumably be modified to compare velocities at 45°N and 45°S. For Hill *et al.* to observe such a mode it would be necessary to observe the absolute position of the limb with respect to the other stars. This is more difficult than their present experiment, but may be possible in a year or so.

The driving of the oscillations must also be studied, for this can provide further diagnostic information just as the quality factors of seismic oscillations yield information about the dissipative properties of the Earth. Several studies of the growth or decay of normal modes of oscillation of the Sun have been reported^{25, 28–30}, but the results are inconclusive. It is likely that the convection zone in the outer envelope is the seat of the excitation, because that is where the amplitudes of the oscillations are greatest. Spiegel³¹ pointed out that the driving of non-radial p modes may take place in superadiabatic regions: this seems to be quite effective in the thin superadiabatic layer near the top of the zone for modes with high l , though the κ -mechanism, which drives the pulsations of Cepheids and RR Lyrae stars, is more important (H. Ando, and Y. Osaki, unpublished). It is likely that there is a superadiabatic region of much larger extent near the bottom of the convection zone, as is exhibited by models with mixing lengths that never exceed the distance from the nearest boundary of the convection zone³² and is suggested by modal calculations of convection zones in A stars (J. Toomre, J.-P. Zahn, J. Latour, and E. A. Spiegel, unpublished). This may contribute towards driving modes with lower l . The direct influence of the convection itself is also likely to be important³³, both in the way it modulates the heat flux and by the direct action of the Reynolds stresses. This presents a severe theoretical difficulty, as does the treatment of the reflection and transmission characteristics of the base of the corona. Indeed, these oscillations may be the principal source of energy maintaining the corona. It is important to inquire whether the continual interplay of these processes maintain the oscillations at approximately constant amplitude and phase, or whether, as some observations suggest^{20, 22, 36}, the oscillations are triggered by occasional violent events such as flares, and then decay. If the former is the case, nonlinear pro-

cesses will determine the amplitudes; if the latter, it might be possible to approximate the decay using linear theory. In either case resonant interactions between modes may be important, and may explain why a particular mode is picked out from the closely spaced g mode spectrum. But whatever is occurring, the observed amplitudes will provide additional information that sooner or later will lead us to a better understanding of the Sun.

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Observation of free oscillations of the Sun

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The Fraunhofer absorption lines for potassium and sodium on the Sun are compared with the corresponding lines in the laboratory using a resonant optical scattering method. The observed shifts between the Sun and laboratory lines may be interpreted in terms of the gravitational redshift (GRS), motion of the laboratory relative to the Sun and oscillatory terms which may be related to oscillations of the Sun.

IN 1961, one of us proposed a new method for solar observation¹, which has since been applied successfully^{2–4}. The method is best illustrated with reference to Fig. 1: A circularly polarised absorption line, represented by the solid upper curve *a*, is incident on a K or Na vapour placed in a longitudinal magnetic field of such a strength as to place the anomalous Zeeman components, curve *b*, near the steepest parts of the solar absorption line. The intensity of resonantly scattered

light of left-handed polarisation is given by N_L whereas that for right-handed polarisation is given by N_R . It is clear from the figure that when there is no relative displacement between the solar and laboratory lines, N_L equals N_R . If, however there is a relative shift between the two lines, as illustrated by the dotted upper curve *a'*, the two intensities are no longer equal. Since the narrow laboratory Zeeman components overlap the steepest parts of the broader solar line, this method is extremely sensitive to small shifts.

Apparatus

The apparatus was developed in Birmingham during 1973, and taken to the observatory at the Pic-du-Midi in France during the autumn of 1974, where the present data were recorded. Unfortunately, of the 12 days on which observation was possible, only 2 yielded data of sufficient quality and duration to allow

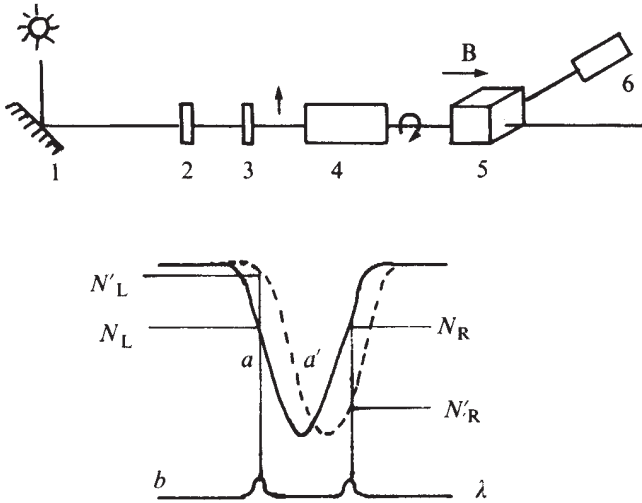


Fig. 1 Upper: Schematic view of apparatus (see text). Lower: Principle of apparatus showing solar absorption line *a* and the position of the normal Zeeman components *b*. The dotted curve *a'* is for a shifted line.

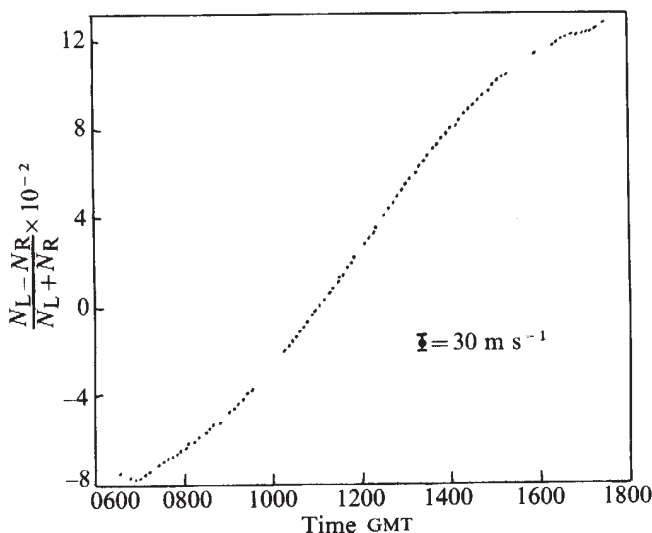
for analysis. A small equatorially mounted servo controlled heliostat (1) directs integral solar light, appropriately filtered by a thermostatically controlled interference filter, (2) through a polariser (3) and electro-optical light modulator (4) which produce the requisite left- or right-handed polarisation, by the application of the appropriate electric potential. The light thus enters a vapour cell (5) situated in the field of a permanent magnet; the resonantly scattered light is detected by a cooled photomultiplier (6), and the processed output pulses are recorded on digital magnetic tape for subsequent computer analysis.

Integral solar light was used to reduce random shifts from the motion of individual granules of the Sun, the five-minute oscillation, and differential shifts across the rotating solar disk. Displacements caused by imprecise guiding and atmospheric turbulence also become less serious.

Results

The recorded data thus consist of numbers of counts N_L and N_R , taken at approximately 0.5-s intervals. The difference between these data points over their sum $[(N_L - N_R)/(N_L + N_R)]$ then gives a measure of the line shift corrected for any intensity

Fig. 2 Data obtained on September 27, 1974 using a K cell.



fluctuation. Each data point consisted of $\sim 5 \times 10^5$ counts, and the mean of 20 such ratios were taken, rejecting any pair which showed a gross deviation from that mean. Thus data points were obtained at 20-s intervals with a statistical accuracy of $\sim 2 \times 10^{-4}$ which corresponds to a shift of $\sim 1 \text{ m s}^{-1}$. Results obtained on September 27, 1974 are shown in Fig. 2.

Analysis

The observed shift of the solar absorption line relative to the laboratory line may be expressed in terms of velocities as

$$V_{\text{obs}} = V_{\text{orbit}} + V_{\text{spin}} + V_{\text{GRS}} + V(t) \quad (1)$$

where

$$V_{\text{orbit}} = (\mathbf{V}_{\odot} - \mathbf{V}_{\text{E}}) \cdot \mathbf{r}$$

is the resolved component of the relative velocity of the Sun and Earth along the unit radius vector $\mathbf{r}$ joining their centres, with due allowance for planetary and lunar perturbations⁵. The observer's velocity, at a time t , relative to the Earth's centre along $\mathbf{r}$ is given by

$$V_{\text{spin}} = \mathbf{V}_{\lambda} \cdot \mathbf{r} = \omega_{\text{E}} R_{\text{E}}(\lambda) \cos \lambda \cos \delta \sin \frac{\pi}{12} (t - t_0) \quad (2)$$

where ω_{E} is the angular velocity of the Earth, $R_{\text{E}}(\lambda)$ is the observer's distance from the centre of the Earth, λ the latitude, t_0 is local noon and δ is the declination of the Sun.

The gravitational redshift is

$$V_{\text{GRS}} = \frac{GM_{\odot}}{c} \left(\frac{1}{R_{\odot}} - \frac{1}{R_{\odot\text{E}}} \right) - \frac{GM_{\text{E}}}{cR_{\text{E}}}$$

+ (terms due to second-order Doppler shifts)

where $M_{\odot}$, M_{E} , $R_{\odot}$ and R_{E} are the Sun and Earth masses and radii respectively, and $R_{\odot\text{E}}$ is the distance from the Earth to the Sun. The gravitational constant is given by G and the velocity of light by c .

Finally,

$$V(t) = V_0 + V_1(t)$$

where V_0 represents time-independent shifts due to, for instance, collision effects in the Sun, and $V_1(t)$ contains time-dependent terms such as solar oscillations which form the basis for this report.

The terms V_{orbit} and V_{GRS} in equation (1) may be regarded as essentially constant during any one day, whereas the V_{spin} term varies sinusoidally as illustrated by equation (2). The amplitude of this term is $\sim 340 \text{ m s}^{-1}$ during the autumn at Pic-du-Midi.

The data of Fig. 2 clearly indicate the sinusoidal variation and provide an accurate calibration of the apparatus in terms of known parameters. It is further clear that the data pass through zero, indicating that at this time the solar absorption line and the laboratory line have a zero relative shift. Thus

$$-V_{\text{GRS}} = V_{\text{orbit}} + V_{\text{spin}} + V(t)$$

If it is assumed that $V_1(t)$ terms are small or may be averaged out, then the gravitational redshift may be determined directly from this 'null' experiment in terms of known parameters. Details of this measurement will be reported elsewhere.

To investigate the $V_1(t)$ terms, a two-parameter fit of the form

$$a + b \sin \frac{\pi}{12} (t - t_0)$$

was made to the data. The residuals, representing the differences between this fitted curve and the actual data points, were then found and are shown in Fig. 3 for the data obtained on September 27 using a K vapour cell. These data clearly suggest an

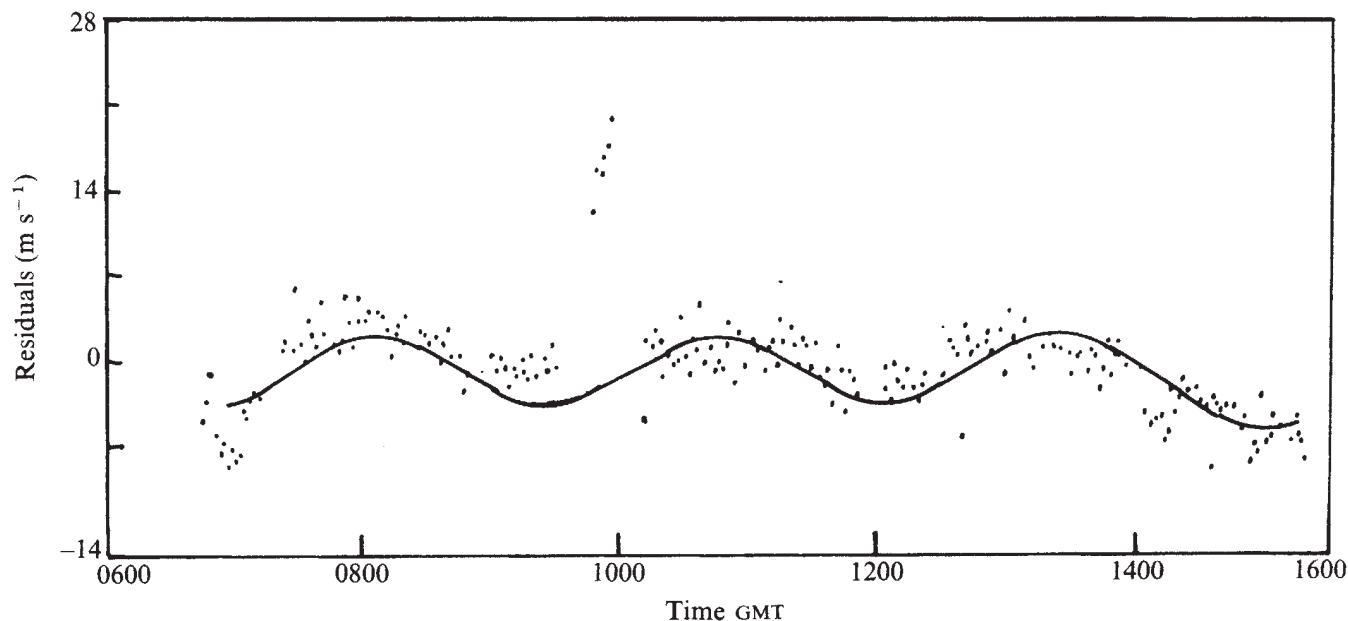


Fig. 3 Plot of residuals showing fitted 2.65-h oscillation.

oscillatory form for $V_1(t)$ with a period between 2 and 3 h. A least squares fit to these points yielded a sine wave of amplitude $2.70 \pm 0.24 \text{ m s}^{-1}$ and a period of $2.65 \pm 0.04 \text{ h}$. Similar data, but of lower quality, obtained on November 2 using a Na vapour cell gave an amplitude of $4.5 \pm 0.7 \text{ m s}^{-1}$ for an oscillation of period $2.7 \pm 0.1 \text{ h}$.

The data of September 27 were further subjected to an autocorrelation and power spectrum analysis using a Hamming window. The resultant power spectrum, see Fig. 4, demonstrates clearly the existence of the 2.65-h period and suggests the presence of two or three further peaks whose amplitudes and periods are given in Table 1.

Interpretation

The observed oscillations could be due to oscillations of the Sun or may be attributed to other effects. These other effects include the terrestrial atmosphere, nonlinearities in the apparatus, poor servo guiding, local voltage fluctuations, temperature instabilities and several more detailed instrumental effects. These causes, however, have been rejected after careful consideration of the types and magnitudes of variation that they might produce.

It is thus suggested that the 2.65-h period be identified with the gravest radial mode of oscillation of the Sun, the 58-min period with the fundamental non-radial mode and the 40-min

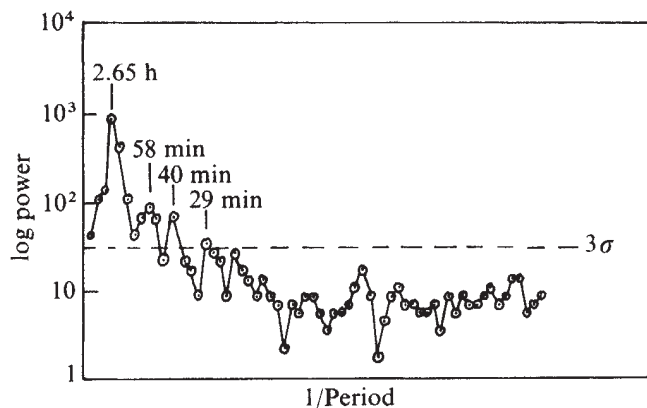
period with the second harmonic of the radial mode. The remaining peak in the power spectrum at 29 min can be traced to instrumental effects associated with the heliostat drive.

Extrapolation of the period-density relation of various pulsating stars⁶, if taken as a rough guide, places the expected period (P_0) for the Sun $1 \text{ h} < P_0 < 4 \text{ h}$. Theoretical models of pulsations⁷⁻⁹ indicate a similar, but narrower, range for the gravest radial mode, with the fundamental period P_0 decreasing from 2.78 h to $< 1 \text{ h}$ as the central density of the Sun increases. Current solar models⁹ predict a period of $\sim 1 \text{ h}$ corresponding to a steep density increase in the solar interior, in marked contrast to the observed 2.65-h period, which is consistent with a nearly homogeneous model of the Sun.

Table 1 Power spectrum of observed observations

Period	Observed amplitude (m s^{-1})	Period predicted by homogeneous model
2.65 h	2.7	2.78 h: radial, fundamental
58 min	0.8	59 min: non-radial, fundamental
40 min	0.7	47 min: radial, second harmonic or 42 min: non-radial, second harmonic
29 min	0.5	(instrumental)

Fig. 4 Logarithmic power spectrum showing the four peaks above the 3σ confidence level.



The value of P_0 depends on the local velocity of sound, $(\gamma k T / m)^{1/2}$, integrated over the Sun, and if γ and m are presumed to be known, P_0 becomes a measure of T , the mean temperature. The present value of P_0 , assuming $\gamma = 5/3$ and the usual composition for the Sun, yields a lower value for T , which in turn would produce a lower neutrino flux^{10,11}.

If indeed the 58-min line is the fundamental of a non-radial mode of oscillation, then this line should be split by the rotation of the Sun¹². Since the degree of splitting increases with the speed of rotation of the interior and since there is no evident splitting of the observed 58-min line, it may be concluded that the interior of the Sun rotates more slowly than once every 12 h.

The peak in the power spectrum corresponding to a 40-min period, is identified with the second harmonic of the radial mode. Oscillations of this period have been previously detected¹³ and were also found with our apparatus during short bursts of good visibility at Birmingham during the spring of 1974.

The somewhat higher amplitude of the oscillations of November 2 for Na atoms, compared with that for K atoms on

September 27, is semi-quantitatively in agreement with expectations for a steady oscillation over this time interval. Sodium atoms in their ground state, being more abundant than potassium atoms, provide unit optical depth at a lower density in the solar photosphere, at which an unattenuated sound wave would have a higher velocity amplitude.

Each observation, taken separately, suggests that the damping is small ($Q > 10$). If it is assumed that the oscillation observed on November 2 is a continuation of that first seen on September 27, and that no substantial excitation occurred during the intervening time, then Q becomes > 300 , consistent with theoretical expectations¹⁴. The total energy content for the fundamental mode of oscillation integrated over the entire Sun is $\sim 10^{31}$ erg, comparable with the energy of a major solar flare. In view, of the large Q for this mode of oscillation, however, the actual energy dissipated is negligible by solar standards.

The data could also be explained in terms of the high order g modes of non-radial oscillation¹⁵. These are predominantly horizontal and can have very long periods. It is difficult, however, to understand why other high order modes have not been detected unless some preferential excitation mechanism exists.

Hill¹⁶ has reported the detection of solar oscillations during his measurements on solar oblateness. The periods reported are all < 1 h and the amplitudes at least an order of magnitude greater than those reported here. It seems difficult to reconcile these data with our work, unless the oscillations are of high

order, so that averaging over the whole solar disk reduces their velocity amplitude substantially.

Improvements in the measurements may help to cast some light on the internal rotation of the Sun and thereby on the Einstein and Dicke theories of gravitation¹⁷. An extension of the work using a two-dimensional spectrometer capable of measuring wavelength shifts across the solar disk may well inaugurate a new field—the probing of the Sun's interior with seismic waves, analogous to its terrestrial counterpart¹⁸.

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Presence of gene for β globin in homozygous β_0 thalassaemia

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In one southern Italian and one Pakistani patient with homozygous β_0 thalassaemia in which no detectable β -globin synthesis occurs and no β -globin messenger RNA is found, the gene for β globin has been shown to be present using complementary DNA. This demonstrates that for these patients the imbalance in chain synthesis is not attributable to a gene deletion.

THALASSAEMIA is a common hereditary anaemia which is caused by imbalanced synthesis of α and β globin chains¹. In α thalassaemia there is reduced synthesis of α globin, which in at least some cases results from a partial or complete deletion of the α -globin genes^{2–4}. Other cases of α thalassaemia are caused by chain termination mutants for which the globin chain is synthesised in very small amounts⁵.

β -Thalassaemia is a more complex hereditary condition, since the decrease in β -globin synthesis can be compensated by increased synthesis of δ and γ -globin chains (as in β_0 thalassaemia) or γ -globin chains only (as in $\delta\beta_0$ thalassaemia). In cases

of β^+ thalassaemia low levels of β -globin synthesis are observed, whereas in β_0 thalassaemia no β -chain synthesis occurs and only haemoglobins A_2 (HbA₂) and F (HbF) are found in the peripheral blood. In some cases of β_0 thalassaemia, messenger RNA (mRNA) for β globin is absent as judged by hybridisation to complementary DNA (cDNA) prepared using viral reverse transcriptase⁶ and also by translation in cell-free protein synthesis systems⁷. In one form of β_0 thalassaemia, from the Ferrara region of Italy, β -globin mRNA (mRNA _{β}) is present in erythroid cell cytoplasm but is not translated⁸.

We have isolated DNA from spleens of two patients with homozygous β_0 thalassaemia. In neither case was there any demonstrable synthesis of β globin in the peripheral blood, nor could mRNA _{β} be detected in the cytoplasmic RNA of spleens or peripheral blood cells. In both cases, however, the hybridisation of cellular DNA to a DNA enriched in sequences specific for β -globin genes (cDNA _{β}) was identical to that of normal DNA, demonstrating the presence of substantially intact genes for β globin in homozygous β_0 -thalassaemic patients.

This confirms our previous report of the presence of β -globin genes in the genetically more complex double heterozygote for

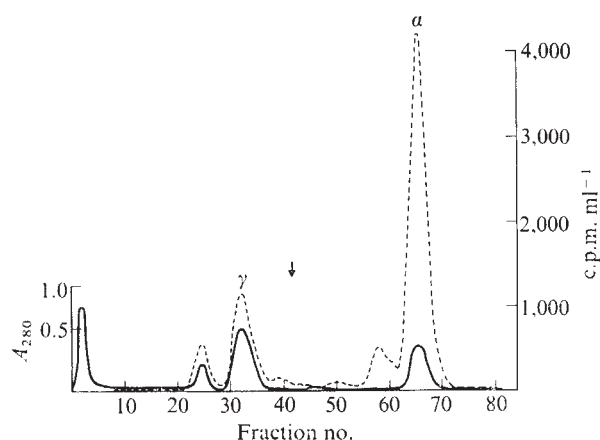


Fig. 1 Globin synthesis in homozygous β_0 thalassaemia. Washed red blood cells from the Pakistani patient with homozygous β_0 thalassaemia were incubated in the presence of ^3H -leucine (specific activity $40\text{--}60\text{ Ci mmol}^{-1}$, Radiochemical Centre) as described previously¹⁵. Extracted whole-cell globin was chromatographed on carboxymethylcellulose¹⁰. Aliquots of the column fractions were monitored for absorbance at 280 nm and for radioactivity. Essentially no radioactivity (-----) associated with absorbance (—) was seen in the β -globin region (arrow: β -globin peak fraction from parallel marker run). Identical results were obtained with red blood cells from the Italian patient.

$\beta_0/\delta\beta_0$ thalassaemia, for which at least one of the haploid genomes was shown to contain a largely intact β -globin gene⁹. It remains to be determined whether the small deficit in β -gene hybridisation shown for that genome is due to a partial deletion in the haploid $\delta\beta_0$ -globin gene region.

Spleens were removed for therapeutic reasons from two patients with β_0 thalassaemia, for both of whom genetic data confirmed the haematological diagnosis. One patient was of southern Italian origin and the other from Lalipur, Pakistan. In both cases incubation of peripheral blood with ^3H -leucine followed by globincha in separation on carboxymethylcellulose columns in 8 M urea-mercaptoethanol-phosphate buffer systems, pH 6.7 (ref. 10) showed α - and γ -globin chain synthesis only. No β -globin synthesis was observed (Fig. 1, Pakistani patient; identical data were obtained from the Italian patient). In both cases, both parents showed elevated levels of HbA_2 .

Nuclear DNA was prepared from spleen using hydroxylapatite-urea as described previously^{2,11}. Normal DNA was prepared either from non-thalassaemic spleen or from normal placenta. The DNA was fragmented to approximately 200–300 nucleotides in length either by sonication as described previously¹¹ or by boiling for 20 min in 0.3 N NaOH¹².

Normal adult human globin mRNA ($\text{mRNA}^{\alpha,\beta}$) was prepared from reticulocyte-rich blood from a patient with a non-thalassaemic haemolytic anaemia. Cord exchange transfusion blood was obtained from newborn infants with Rhesus incompatibility, and used to prepare $\text{mRNA}^{\alpha,\gamma,\beta}$. α -Thalassaemia globin mRNA (mRNA_{HbH}) was obtained from blood from a patient with haemoglobin H disease^{2,9,13}. Total β_0 -thalassaemia cytoplasmic RNA ($\text{RNA}^{\beta_0\text{thal}}$) was prepared by phenol-chloroform extraction from β_0 -thalassaemic spleen postnuclear supernatant⁹.

cDNA was prepared from the various mRNAs as template, using oligo(T) as primer with avian myeloblastosis virus reverse transcriptase as described previously¹⁴. The labelled nucleoside triphosphate used was ^3H -dCTP (specific activity $25\text{--}27\text{ Ci mmol}^{-1}$; Radiochemical Centre).

All cDNAs prepared from mRNAs hybridised back to their respective templates showing approximately 5–10% apparent double-stranded material in the absence of RNA and 10–15% material which did not hybridise even in great mRNA excess.

Complementary DNA enriched in sequences complementary to mRNA_β was prepared by annealing cDNA_{HbH} , which is already rich in cDNA_β , to the cytoplasmic mRNA from the

previously described case of $\delta\beta_0/\beta_0$ thalassaemia, which contains no detectable mRNA_β sequences. The non-hybridising sequences (cDNA_β) do not bind to hydroxylapatite in 0.12 M sodium phosphate buffer, and were collected, concentrated and characterised as described previously⁹.

Three cDNA_β probes were used in the experiments described below. cDNA_{HbH} was approximately 80% cDNA_β sequences. cDNA_β cycled through hydroxylapatite as described above was >80% pure in one case and >90% in the other, as judged by hybridisation to $\delta\beta_0/\beta_0$ -thalassaemic total cytoplasmic RNA⁹. Both cDNA_β preparations showed plateau levels of approximately 70% when hybridised to a large excess of mRNA which contained β -globin. This reduction in plateau level was due to enrichment of non-hybridising material during hydroxylapatite purification of these cDNA_β (ref. 9).

No β -globin mRNA was detected in the spleen cytoplasmic RNA from each patient (Figs 2 and 3). At a 1×10^6 -fold

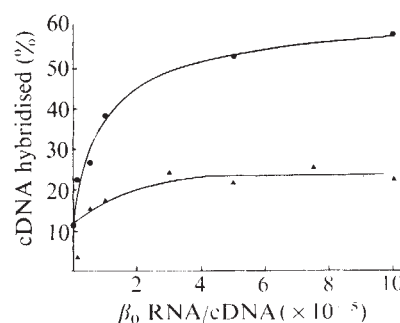


Fig. 2 Hybridisation of β_0 spleen (Pakistani patient) total cytoplasmic RNA with $\text{cDNA}_{\alpha\beta\gamma}$ and cDNA_β . Total cytoplasmic RNA from β_0 spleen was prepared from the post-nuclear supernatant of spleen homogenate by phenol-chloroform extraction⁹. mRNA was prepared by affinity chromatography with oligo(dT)-cellulose (Collaborative Research) from total RNA prepared by phenol-chloroform extraction of foetal, and HbH disease, whole red blood cells. DNAs were prepared to these mRNA templates, using RNA-dependent DNA polymerase from avian myeloblastosis virus (AMV), as described previously^{9,14}. ^3H -dCTP (Radiochemical Centre) of specific activity 27 Ci mmol^{-1} was the only labelled nucleotide present. Specific activity of the cDNAs was $16,100\text{ c.p.m. ng}^{-1}$ at a counting efficiency of 38%. cDNA_{HbH} was used to prepare cDNA_β , by hybridisation to a 10,000-fold excess of $\delta\beta_0/\beta_0$ total cytoplasmic RNA, followed by isolation of the unhybridised, single-stranded fraction by chromatography on hydroxylapatite⁹. Purity of cDNA_β was estimated to be >80% (see text). A constant amount of 0.05 ng $\text{cDNA}_{\alpha\beta\gamma}$ or cDNA_β was hybridised with increasing amounts of β_0 -spleen cytoplasmic RNA, in a volume of 2 μl 50% formamide, 0.5 M NaCl, 25 mM HEPES buffer, pH 6.8, 1 mM EDTA, containing 2.5 mg ml^{-1} *Escherichia coli* RNA. Samples were sealed in silicone-coated glass capillaries, and incubated at 43°C for 168 h. Percentage of cDNA which had hybridised was determined by assay with the single-stranded specific nuclease, S1, followed by perchloric acid precipitation of hybridised material. Hybridisation of $\text{cDNA}_{\alpha\beta\gamma}$ (●) and cDNA_β (▲) to increasing amounts of β_0 -spleen cytoplasmic RNA is shown. Both cDNAs contain a double-stranded component of approximately 11%. Hybridisation of both cDNAs, in the same conditions, to a 1×10^6 -fold excess of *E. coli* RNA, still gave values of 11% for the double-stranded component.

excess of β_0 -cytoplasmic RNA, cDNA_β and cDNA_{HbH} hybridised to approximately 12% above background, indicating a virtual absence of β -globin sequences. The low level of hybridisation of the β -enriched cDNAs is attributable to contaminating α - or γ -globin sequences in the cDNA hybridising to their complementary sequences in the β_0 -cytoplasmic RNA.

In contrast, α - and γ -globin sequences were readily detected by the $\text{cDNA}_{\alpha\beta\gamma}$ (Fig. 2) and $\text{cDNA}_{\alpha\beta}$ (Fig. 3) in both preparations of spleen β_0 -cytoplasmic RNA at RNA-cDNA ratios as low as 1×10^5 . The fact that $\text{cDNA}_{\alpha,\beta}$ and $\text{cDNA}_{\alpha,\beta,\gamma}$

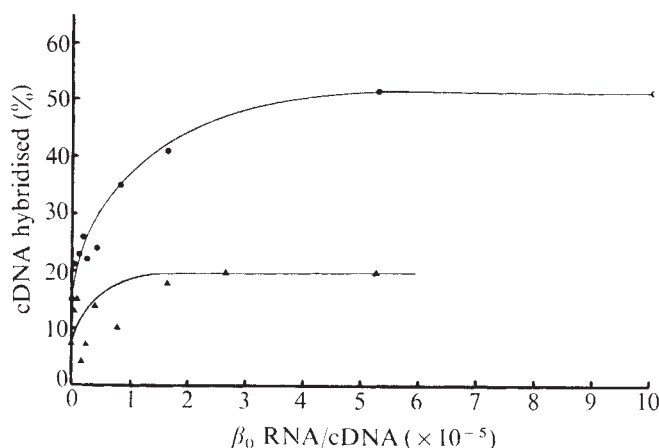


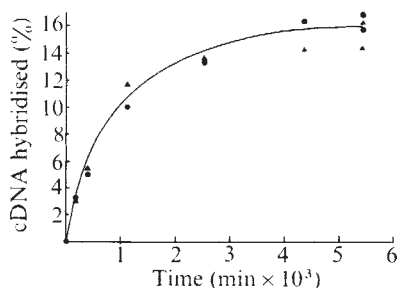
Fig. 3 Hybridisation of β_0 -thalassaemic spleen (Italian patient) total cytoplasmic RNA with cDNA_{HbH} and cDNA _{α , β} . β_0 -spleen cytoplasmic RNA, cDNA_{HbH} and cDNA _{α , β} were prepared as described in Fig. 2. cDNA_{HbH} was estimated to contain 80% β -globin sequences⁹. A constant amount (20 pg) of cDNA_{HbH} (Δ) or cDNA _{α , β} ($\bullet$) was hybridised with increasing amounts of β_0 -spleen cytoplasmic RNA in 1 μ l hybridisation buffer at 43°C for 10 d. Proportion of cDNA rendered resistant to S1 nuclease digestion was determined. Both cDNA_{HbH} and cDNA _{α , β} hybridised to 80–85% to their respective templates.

do not hybridise to levels greater than 50–60%, however, is further evidence for the absence of β -globin sequences.

Hybridisation to total normal and β_0 -thalassaemic DNA was carried out in cDNA _{β} excess. Figure 4 shows the rate of reannealing of the cDNA_{HbH} to the DNA from the Pakistani patient. The curves for normal and β_0 -thalassaemic DNA are essentially identical.

To demonstrate with greater rigour the equivalence of the thalassaemic and normal DNAs, the extents of hybridisation were determined for both cases at a number of different cDNA–DNA ratios. At low ratios of cDNA _{β} –total DNA, the effects of contaminating α - and γ -globin cDNA sequences will be greatly reduced. At ratios varying from 6 pg cDNA _{β} to 100 μ g total DNA to 70 pg cDNA _{β} to 100 μ g total

Fig. 4 Hybridisation of excess cDNA _{β} to normal and β_0 DNA (Pakistani patient). DNA was prepared from normal and β_0 spleen, and fragmented to a mean size of 200–300 nucleotides single-stranded length, by sonication, as described previously^{2,11}. Samples of 2.16 mg normal, or β_0 DNA, and 0.5 ng cDNA _{β} were lyophilised and dissolved in 0.12 M sodium phosphate buffer, pH 6.8, at a DNA concentration of 5 mg ml⁻¹. Aliquots (43 μ l) (one-tenth total volume) were sealed in silicone-treated glass capillaries, boiled for 10 min, and incubated for various times at 60°C. At selected times, contents of capillaries were expelled with 210 μ l S1 nuclease assay buffer and stored at –20°C until assayed with S1 nuclease to determine the proportion of cDNA which had hybridised⁹. A background S1 resistance of approximately 2% has been subtracted, and percentage hybridisation has been calculated relative to the maximum amount of hybridisable cDNA _{β} (70%). $\bullet$, Percentage cDNA _{β} hybridised to normal DNA; Δ , percentage cDNA _{β} hybridised to β_0 DNA.



DNA the extent of hybridisation is the same for the normal and the two thalassaemic DNAs (Fig. 5).

Analyses of both thalassaemic DNAs with cDNA _{α , γ , β} in excess demonstrated a complexity of the total globin genes identical to that of normal DNA⁸.

We have previously demonstrated cross hybridisation between β -globin and δ -globin gene sequences⁹. It is known from genetic¹ and molecular⁹ analyses that there are single copies of each of these genes, and a deletion of the β -globin gene would be apparent as a 50% reduction in extent of hybridisation and greater than 50% reduction (because of mismatching) in rate of hybridisation. The identity of the hybridisation for normal and thalassaemic DNAs demonstrates that no substantial deletion of the β -globin gene has occurred.

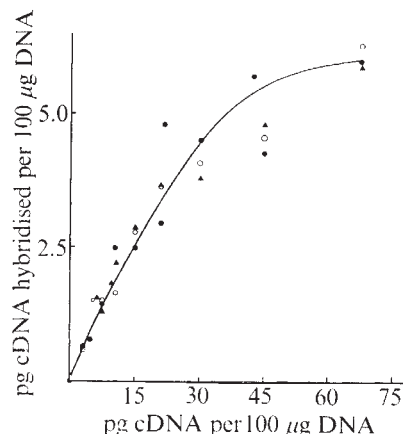


Fig. 5 Hybridisation of cDNA _{β} to normal and β_0 DNAs as a function of cDNA–DNA ratio. Normal or β_0 -thalassaemic DNA was prepared from spleens as described in Fig. 3, except that fragmentation to 200–300 nucleotides was achieved by boiling in 0.3 N NaOH for 20 min. cDNA _{β} (>90% pure) was mixed at the indicated ratios with normal or thalassaemic DNA (50–250 μ g) in 0.12 M sodium phosphate buffer, pH 6.8, 1 mM EDTA, at a DNA concentration of 5 mg ml⁻¹. Mixtures were sealed in capillaries, heated to 100°C for 10 min, and incubated at 68°C for 5,200 min. Samples were diluted with S1 nuclease assay buffer (500 μ l per 100 μ g DNA) and assayed with S1 nuclease to measure the proportion of cDNA hybridised⁹. A background S1 resistance of 2% has been subtracted, and the percentage of cDNA _{β} hybridised has been calculated relative to the maximum amount of hybridisable cDNA _{β} (70%). Results are shown as pg cDNA _{β} hybridised per 100 μ g DNA as a function of cDNA–DNA ratio. $\bullet$, Normal DNA; $\circ$, β_0 -thalassaemic DNA (Italian patient); Δ , β_0 -thalassaemic DNA (Pakistani patient).

Since the cDNA _{β} used in these experiments is not a complete copy of the mRNA _{β} , a deletion near to the initiation region of transcription, to the 5' end of the mRNA, would be more difficult to detect. A deletion in a non-transcribed portion of the genome which might still be involved in the control of transcription could not be detected using these techniques.

β_0 thalassaemias from other areas (for example, Ferrara⁸ and of Chinese origin¹⁶) contain globin mRNA sequences which are untranslated. This result for homozygous β_0 thalassaemia from two separate geographical locations is in marked contrast to the previous demonstration that a gene deletion is the primary cause of south-east Asian homozygous α_0 thalassaemia. It is still not clear whether transcription of the β -globin RNA sequence is completely absent, either because of a mutation in the initiation region for transcription or a mutation of a necessary control element, or if transcription occurs but the mRNA _{β} sequences are either not processed from nucleus to cytoplasm or are broken down very rapidly without translation.

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letters to nature

Transient X-ray source A1118—61

THE transient X-ray source Ariel 1118—61 was discovered late in 1974 (refs 1, 2). It has been suggested³ that this source may be associated with the long period Mira-type variable RS Cen, which is located within the error box for the X-ray source. The proposed mechanism of X-ray generation was accretion on to a compact object in orbit around the variable star. The periodic variation in the radius of the star would lead to a variable accretion rate at the orbit of the compact object, and give rise to a variable X-ray flux. This suggestion has two important consequences. First, it raises the possibility that some, at least, of the apparently transient X-ray sources can be explained by similar phenomena. For instance, Barnden and Francey⁴ and Shukla and Wilson⁵ reported the presence of two presumably 'transient' X-ray sources in the constellation Cetus. The error box for each source (Cetus X-1 (ref. 4) and Cetus X-2 (ref. 5)) was large, ~ 10 – 15° diameter, but they did just overlap. Of significance here is the fact that the overlap area is centred on O Ceti (Mira), the type star of this class of variables. The two observations were at similar phases (0.12 and 0.22) of the variable, but separated in time by ~ 1 cycle. The difference in phase of the Centaurus and Cetus transients is easily explained by differences in orbit size and expansion velocity.

The second consequence of Fabian *et al.*'s proposed identification is a clear, experimentally testable prediction, that the X-ray source should be observable at intervals of ~ 164.5 d after the outburst observed by Ariel V.

We have used the collimated proportional counter on board Copernicus to test this prediction. The detector was pointed at A1118—61 on June 8, 1975 between 17 h 9 min and 22 h 15 min, a time corresponding to an eclipse of the nearby binary X-ray source Cen X-3. No signal was detected above background during the whole of the observing session. Our 90% confidence upper limit to the source strength in the energy band 2.5–7.5 keV is 3×10^{-11} erg cm⁻² s⁻¹; this is 1.7% of the peak flux reported by Ariel V in the same energy band. Thus it is likely that the X-ray source A1118—61 is not connected with RS Cen, unless for some reason the X-ray generation was turned off during the time when Copernicus was observing the source.

Copernicus observed Cen X-3 on January 30, 1974 precisely 2 periods of RS Cen before the peak of the outburst reported by Ariel V. A1118—61 was well within the field of view of the detector, so we have been able to search for a signal from it during the binary down state of Cen X-3. Again, no signal was detected above an upper

limit of 2×10^{-10} erg cm⁻² s⁻¹ ($\sim 10\%$ of peak Ariel V flux) in the energy band 2.5–7.5 keV. Thus there is no strong case for an association of A1118—61 with RS Cen, based on the mechanism suggested by Fabian *et al.*³

We have also searched for X rays from A1118—61, using Copernicus, at a time well removed from the hypothesised critical phase. The observations were made between April 1975 6 d 23 h 31 min and 7 d 3 h 33 min, again during a binary down state of Cen X-3. On this occasion, too, we were unable to detect the source. Our 90% confidence upper limit on this occasion of $\sim 9 \times 10^{-11}$ erg cm⁻² s⁻¹ in the energy band 2.5–7.5 keV is again substantially below the peak Ariel V flux of 1.8×10^{-9} erg cm⁻² s⁻¹.

Our upper limits should clearly be borne in mind in any attempts to explain this source. In particular, Pacini and Shapiro⁶ have suggested that it is the motion of the compact object in an eccentric orbit which periodically brings the compact object close enough to the primary for accretion rates to become appreciable (this scheme has also been suggested by Clark *et al.*⁷ and Davison and Tuohy⁸ to explain the long term behaviour of Cir X-1). Our upper limits modify some of the constraint equations developed by Pacini and Shapiro for the parameters of the proposed binary system. The June 8 upper limit of 1.7% of the peak flux leads immediately to a lower limit of ~ 0.8 for the orbital eccentricity, as compared with the value of 0.4 derived by Pacini and Shapiro. This higher value of eccentricity also does not seem to be excluded by the theory developed by Wheeler *et al.*⁹, though we note that practically any value for the final eccentricity may be predicted, given a suitable (and not impossible) choice of initial conditions.

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New X-ray measurements of the Crab spectrum in the range 26 keV–1.2 MeV

THE Imperial College scintillation detector on Ariel V made measurements of the X-ray spectrum of the Crab Nebula in 1975 April and September. The detector consists of an $8\text{ cm}^2 \times 4\text{ cm}$ CsI(Na) crystal actively collimated to 8° FWHM, and measures X rays in 15 logarithmically spaced energy channels in the range 26 keV–1.2 MeV. The detector axis is inclined 3° to the spacecraft spin axis, which in turn is normally offset by $\sim 3^\circ$ from the source being viewed. Source counts are thus spin modulated, allowing the background to be subtracted. A residual modulation is further removed by changing the direction of the offset to the source and observing the resulting phase change in the modulation. This observational technique ensures all sources of background are eliminated, the modulation characteristics having been previously determined by laboratory calibration.

Counting rates were then deconvolved from the detector response by considering the effects due to window thickness, crystal dead layer and K shell X-ray escape, as well as the

various statistical processes that occur in the detection sequence from the incident photon to the phototube output.

The 1975 observations of the Crab Nebula were first analysed separately, but as no significant variation was observed they have been combined in Fig. 1. Error bars represent 1σ counting statistics, which we believe dominate any other systematic errors. Also plotted on this figure are data below 20 keV obtained from a rocket flight by Toor and Seward¹ in 1970, and results above 1 MeV from balloon flights by Baker *et al.*² in 1972 and Schonfelder *et al.*³ in 1974.

Though there have been many studies of the Crab Nebula by balloon and rocket experiments observing at $< 100\text{ keV}$ (see ref. 2) and some limited observations above 1 MeV, there has previously been a noticeable lack of any accurate observations between these two ranges.

The dotted line shown in Fig. 1 is the best fit given by Toor and Seward to all available data and extrapolated from their limit of $\sim 50\text{ keV}$ up to 10 MeV. As can be seen, the fit to the Ariel V results is excellent up to 500 keV and lies within one s.d. of the final point at 840 keV. Our results are thus consistent with a power law extrapolation to beyond 1 MeV, and hence with Schonfelder's upper limits. On the other hand, if Baker's results are correct, this would imply a rather abrupt hardening of the spectrum at about 800 keV, with a consequent softening above 10 MeV to bring the spectrum into agreement with spark chamber measurements by Kniffen *et al.*⁴ at 50–100 MeV.

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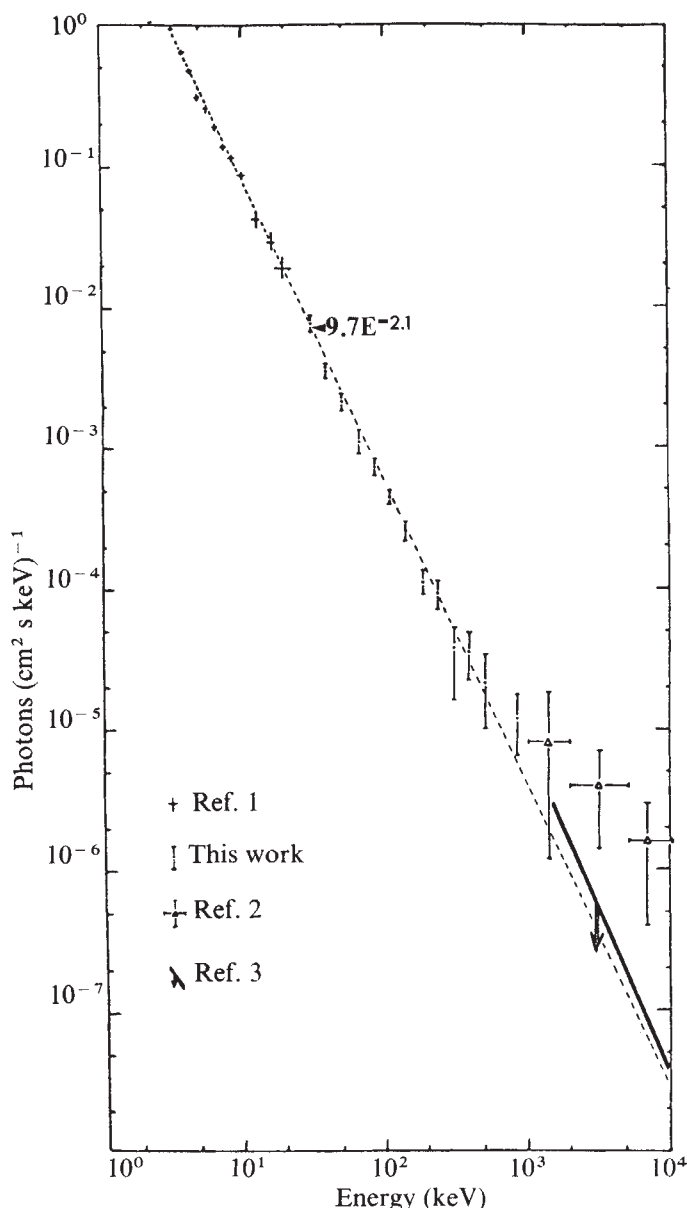
Meteor radar rates and the solar cycle

A WORLDWIDE increase in meteor echo rates in 1963 was observed in New Zealand¹, Canada and Sweden and has been widely discussed in the literature^{2–8}. From radar observations in 1953–66 I reported⁹ a long term variation in the echo count rate with a peak occurring in 1963 near the solar minimum. Those observations also showed that the height of first appearance of meteors from a given shower had remained nearly constant at $\sim 110\text{ km}$, whereas the average endpoint height had risen by $\sim 11\text{ km}$ from 1956 to 1963. It was thus evident that the 1963 peak in the meteor echo count rate was of atmospheric origin. We report here further observations, and propose that the phenomenon can be explained by a solar controlled variation of the atmospheric density gradient at the meteor ablation level, probably caused by a variation in the solar X-ray flux.

My observations were made at the Onsala Space Observatory in the period 1953–72. Visual and radar recordings of meteors have been regularly made in August since 1953. A radar control run in September was operated intermittently from 1953 to 1959 and has been regularly performed since 1962. The radar equipment records echoes of duration $\geq 0.02\text{ s}$ corresponding to meteors brighter than about fifth zenithal magnitude. The experimental technique has been described elsewhere^{5,10}.

Figure 1a depicts the mean night-time hourly rate of meteor radar echoes of all durations, as observed from 1953 to 1972 (1954 is missing and 1971 not yet reduced). A long term variation in the August meteor rate curve is evident. The operational run in August includes the period of maximum activity of the Perseid meteor shower, but the length of the run is such that

Fig. 1 Observations of the Crab Nebula from 1 keV to 10 MeV.



the observed total echo rates may be considered a measure of sporadic meteor activity. It is also evident that total echo rates for the control period in September, when no prominent shower is active, exhibit similar temporal variations. It is therefore concluded that the observed time variation in meteor rates is representative of meteor rates in general. Figure 1 suggests a variation by a factor of ~ 2 in the echo rate curve with peaks primarily confined to times near solar minimum, and further compares the total radar rate curve with the mean endpoint height and trail length of meteors of a given shower. These meteors represent a homogeneous sample with respect to velocity, angle of incidence and composition. It is evident that the meteor endpoint heights and meteor radar rates exhibit similar temporal variations. There is further an inverse relationship between radar rates and the mean trail length of meteors (Fig. 1c). We found that the echo amplitudes showed a temporal variation similar to that of the radar rates.

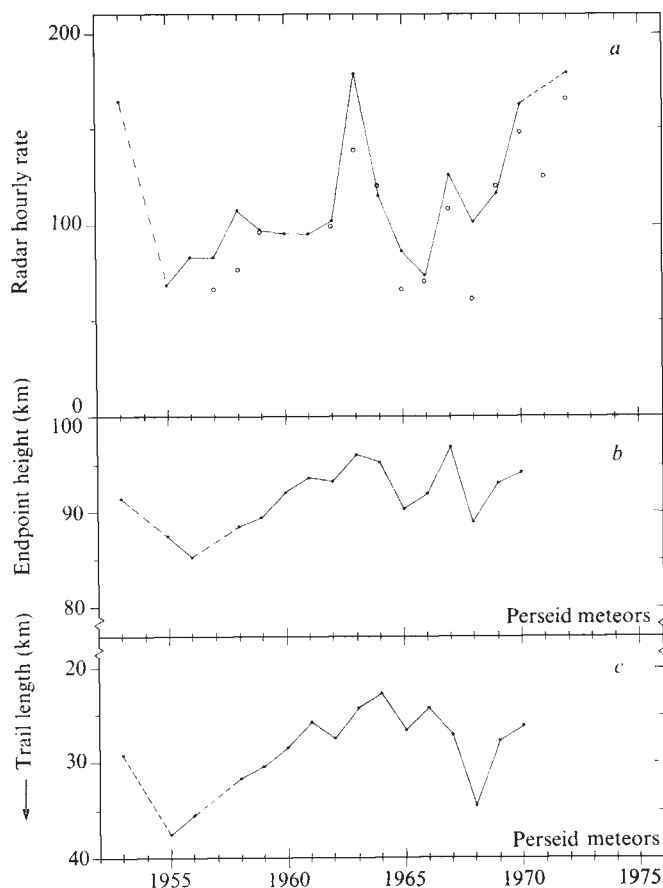


Fig. 1 Comparison between meteor echo rates, meteor end-heights and meteor trail lengths. *a*, Mean night-time hourly rate of meteor radar echoes of duration > 0.02 s observed in August (●—●) and September (○) during the period 1953–72. Radar observations were made at a frequency of 33 MHz. *b*, Mean endpoint height of Perseid meteor trails determined from combined radar and visual observations. *c*, Mean trail length of Perseid meteors.

Figure 1 suggests a dependence of radar rates on position in the solar cycle. The phase of the solar cycle variation is analysed in the periodogram in Fig. 2. The zero epoch in the diagram is that of the sunspot maximum. It is evident that the highest radar rates are observed 4–6 yr after a solar maximum and the lowest, 1–2 yr before a solar maximum. This result is consistent with those of an analysis of visual meteor rates (1844–1933) published by Bumba¹¹. The scale in Fig. 2 may be approximately referred to solar minimum by subtracting 6.5 yr. One may therefore conclude from Fig. 2 that the phase of the radar rate curve is such that peak meteor rates are observed 1–2 yr before a solar minimum.

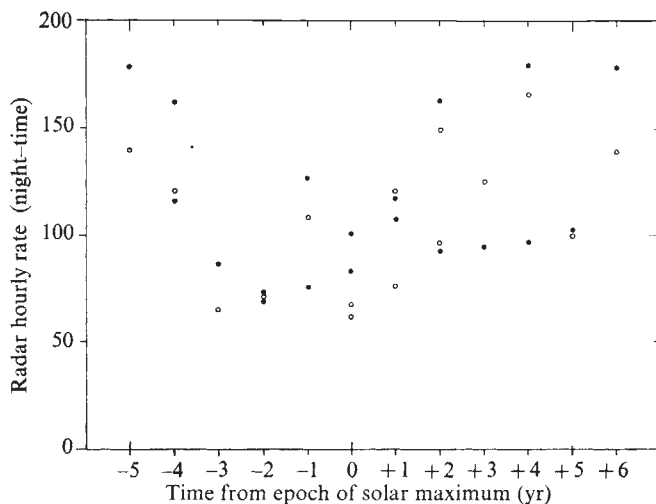
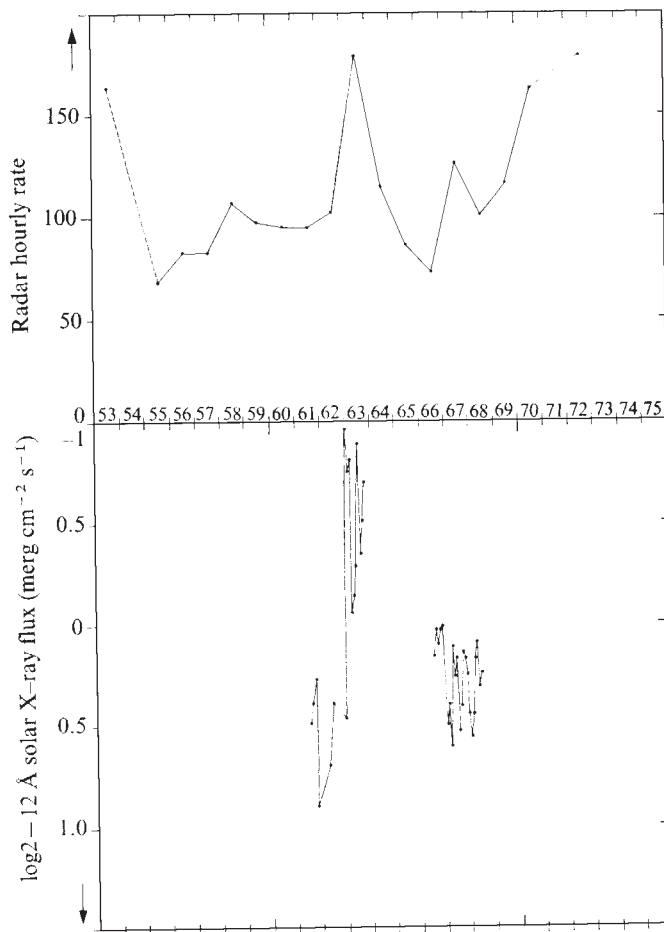


Fig. 2 Variation of meteor radar rates with position in the solar cycle. Epochs of sunspot maxima are 1957.9 and 1968.9. All time differences in the diagram have been rounded off to an integer number of years.

A long term variation in radar rates, meteor trail length and radar echo strength may be qualitatively explained if it is assumed that the density gradient of the neutral atmosphere between 85 and 110 km varies periodically with position in the solar cycle. If the density gradient is increased near solar minimum, the meteor ablation process will occur over a shorter

Fig. 3 Mean night-time hourly rate of meteor radar echoes (upper diagram); mean monthly non-flare 2–12 Å solar X-ray flux after Wende (lower diagram).



length of trail and a larger electron line density will result from a particular meteoroid. This will increase the echo signal strength and thus allow smaller meteors to come within the detection threshold of the radar, thereby producing an increase in the total echo rates.

The observed temporal variation of meteor trail length provides information on the variation of the atmospheric density scale height. The length of a meteor trail may be written¹²

$$L = cH \sec z_R \quad (1)$$

where L and H are meteor trail length and atmospheric scale height, respectively; z_R is the zenith distance of the radiant and c is a numerical constant, which depends on mass, velocity and limiting visual magnitude. Insertion of $H = 5.7$ km, $L = 28.2$ km and $\sec z_R = 1.40$ gives $c = 3.53$. Observed maximum and minimum values of trail length were 37.5 km for 1955 and 22.7 km for 1964. On the basis of the data it is tentatively suggested that the average density scale height at the meteor ablation level has varied from about 7.5 km in 1955 to 4.5 km in 1964.

The heating mechanism required to produce a solar cycle effect in the atmospheric density gradient at the meteor ablation level is not properly understood. A long term variation in the X-ray and particle flux emitted from the Sun seems to be the most probable cause. Kreplin¹³ has summarised the available rocket and satellite data on the solar X-ray flux and found that for wavelengths of 8–20 Å, which penetrate to, and are absorbed in, the meteor zone, the solar cycle variation may exceed a factor of 100. A very variable heat source thus exists in the meteor ablation zone.

Wende¹⁴ has investigated the solar cycle variation in the integral, non-flare, solar X-ray flux between 2 and 12 Å, as measured by the spacecrafts Injun 1, Injun 3, Explorer 33 and Explorer 35. Figure 3 compares the monthly means of X-ray flux with the radar rate curve. Although the X-ray data cover only three periods in 1961–68, the data suggest an inverse correlation between meteor rates and solar X-ray flux. Of particular interest is the rather abrupt decrease in the solar X-ray flux observed at the beginning of 1963 simultaneously with the reported worldwide increase in meteor radar rates. The 1963 increase in meteor radar rates might thus reasonably be ascribed to a change in the atmospheric density gradient due to a sudden drop in the solar X-ray flux.

Existing theories of meteor ionisation generally assume an isothermal atmosphere, and do not take into account spatial and temporal variations in temperature in the meteor ablation zone. Therefore no detailed theoretical explanation of the observed long term variations in meteor radar rates and heights is available. Observed changes in radar echo amplitudes of bright, visual meteors⁹ are far larger than what one would expect from meteor ionisation theory.

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Self reversal of thermoremanent magnetisation in basalts and global lunar magnetism

RYALL and Ade-Hall have shown experimentally¹ that prolonged heating of pillow basalts in suitable conditions results in the exsolution of a 'daughter' phase from the host titanomagnetite. They suggest that in an applied field the daughter phase acquires a chemical remanent magnetisation (CRM) when it grows to the single domain size and that on cooling, the 'mother' or host phase is subjected to an interaction field from the daughter phase, which, they suggest, could outweigh the effect of the external field and result in a net reversed moment of the sample. They suggest that magnetostatic or possibly exchange interaction might be responsible.

By using magnetostatic theory which has already been applied to a simple model of a two-phase titanomagnetite particle—and, on a rather larger scale to the problem of the residual moment of the Moon—it can be shown that a magnetostatic interaction can indeed explain their results.

Consider a simple model of a two-phase particle as shown in Fig. 1. The daughter phase is assumed to be spherical, of radius a , positioned at the centre of a spherical host phase of radius b . If the relative permeabilities are μ_{1r} and μ_{2r} respectively, and if the daughter phase carries a CRM of M_1 per unit volume, where $M_1 = M^*_1 - H_d$ ($\mu_{1r} - 1$), (H_d is the demagnetising field within phase 1, and M^*_1 is the CRM if $H_d = 0$), then the external moment m_e is given by²

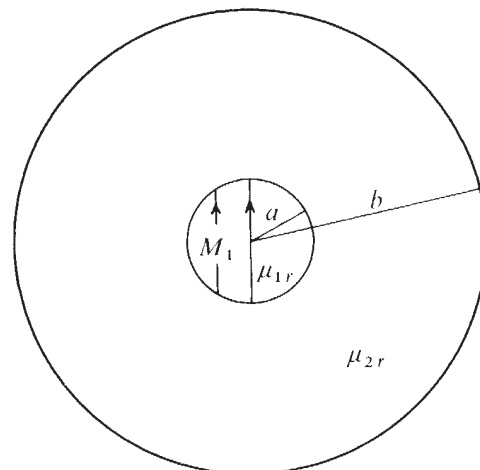
$$m_e D^2 / 4\pi b^3 = 3\sigma \mu_{2r} M^*_1 (\mu_{1r} + 2\mu_{2r})(\mu_{2r} + 2) \quad (1)$$

provided that $\sigma \ll 1$ where $\sigma = (a/b)^3$, and that the permeability of the medium in which the moment is measured (μ_{3r}) is unity. For small values of σ , $D = (\mu_{1r} + 2\mu_{2r})(\mu_{2r} + 2)$. On cooling, the host phase (Fig. 2) will acquire a thermoremanent magnetisation (TRM) in the field produced by the internal dipole (attributable to the CRM) and if k represents the efficiency of the TRM acquisition process (that is, $M_{\text{TRM}} = kH$) then the external moment, attributable to the spherical shell alone³ is m_i where

$$m_i D^2 / 4\pi b^3 = 6k\sigma M^*_1 (\mu_{1r} - \mu_{2r}^2) \quad (2)$$

If $\mu_{2r} = \sqrt{\mu_{1r}}$, the external moment attributable to the host phase is thus zero. If the volume of the host particle is multiplied by $\sim 10^{30}$ the body becomes of planetary dimensions,

Fig. 1 Model of two-phase particle (radii a and b). The inner 'daughter' phase (radius a) carries a CRM/ M_1 (see text). The moment, m_e , of the particle is given by equation (1).



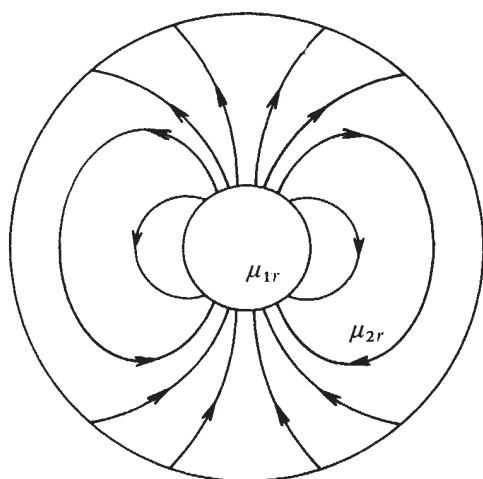


Fig. 2 The component of TRM (schematic) acquired by a host phase in field of a daughter phase. The moment, m_i attributable to this component is given by equation (2). See text for notation.

and if $\mu_{1r} = \mu_{2r} = 1$, then $m_i = 0$. This result corresponds to that derived for the case of the Moon by Runcorn⁴, who showed that if the outer shell of the Moon were magnetised in the field of an internal dipole which has now disappeared, the present lunar dipole moment should be zero. Since μ_{2r} , however, must always exceed unity if a TRM is acquired and probably exceeds μ_{1r} in the Moon, a small negative lunar dipole moment should be observed³.

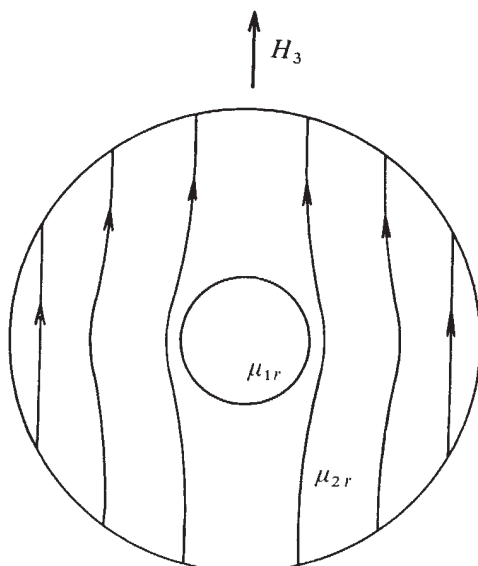
Similar theory applied to the component of TRM which the host phase acquires as it cools in the external field H_3 (Fig. 3), enables the following equation to be derived for the external moment m_e .

$$m_e D^2 / 4\pi b^3 = 3kH_3(\mu_{1r} + 2\mu_{2r})^2 \quad (3)$$

The total moment m of the two-phase particle is thus the sum of three terms given by equations (1)–(3).

The only negative contribution to the moment comes from the second term, and then it arises only if $\mu_{2r}^2 > \mu_{1r}$. This condition is almost certainly fulfilled if, as Ryall and Ade-Hall suggest¹, the CRM resides in a single domain, daughter phase for which μ_{1r} that is, $(dB/\mu_0 dH) = 1$.

Fig. 3 The component of TRM (schematic) acquired by a host phase in an external field H_3 . The moment, m_e , attributable to this component is given by equation (3). See text for notation.



A second condition which must hold if m is to be negative is that $|m_i| > m_e$, and if μ_{2r} is assumed to be $\gg 1$, then $\sigma M^*_{1r} > 2H_3$. A typical titanomagnetite has a spontaneous magnetisation of the order of 10^5 A m^{-1} (100 gauss), so putting $M^*_{1r} (= M_1 \text{ for a single domain grain with } \mu_{1r} = 1)$ equal to 10^5 A m^{-1} and since $H_3 \approx 80 \text{ A m}^{-1}$ (1 oersted), then $\sigma > 1.6 \times 10^{-3}$, which corresponds to a ratio a/b of ~ 0.1 . Thus, only about 0.16% by volume of daughter phase is required to produce reversal.

The third condition which is necessary is that $|m_i| > m_e$; thus for $\mu_{2r} \gg 1$, $k > \mu_{2r}$. To see whether this condition is reasonable it is necessary to consider how the TRM process operates. Before the host phase cools (assumed multidomain) but while it is still below the Curie point, the induced magnetisation, M , at any point is $(\mu_{2r} - 1)H$, where H is the local field, and where, if μ_{2r} is high, $M \approx \mu_{2r}H$. On cooling, this magnetisation becomes blocked in as domain walls become immobilised in local potential wells. On further cooling, the spontaneous magnetisation will change from the value $M_s(T_B)$ at the blocking temperature T_B , to $M_s(T_0)$ at room temperature, T_0 . Thus, $k = \mu_{2r}M_s(T_0)/M_s(T_B)$. Since spontaneous magnetisation usually increases on cooling, then $k > \mu_{2r}$, and that condition can be satisfied. There are, of necessity, many simplifications in this argument. The factor μ_{2r} is assumed to be constant though it will in practice vary with temperature. Each host particle will contain many inclusions of daughter phase which are represented here by a single inclusion which has the same properties as the individual inclusions and a volume equal to the sum of the individual volumes. Spherical symmetry is also assumed. The assumption that $M_{\text{TRM}} = kH$ will break down near the surface of the single domain inclusion since the local field there will be well above the low field range where a linear dependence is normally found. Nevertheless, provided σ is small, the regions where the nonlinear effects operate will also be of relatively small volume and may possibly be neglected.

In spite of all the simplifications, reverse TRM of the type found by Ryall and Ade-Hall can be explained on magneto-static theory using the model described here. Before the daughter phase forms, the moment is given by equation (3). As the daughter phase increases in size through the single domain range, σ increases and the second term (equation (2)), which dominates the first (equation (1)), causes a decrease in moment until reversal occurs. Once the daughter phase exceeds the single domain size, M^*_{1r} decreases and μ_{1r} will increase so that the negative contribution will diminish and the moment will revert to normal, in line with experimental observations¹.

It is interesting that the second term (equation (2)) which governs whether or not reversal occurs is the contribution from that component of remanence in the shell which was produced by the field of the central magnetised inclusion. This would also be the term describing the present lunar global dipole moment were the shell originally magnetised in the field of an ancient dipole which has now disappeared³.

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Origin of corundum-normative intrusive and extrusive magmas

MANY intrusive and extrusive calc-alkaline suites show a continuous trend from diopside-normative basic magma to corundum normative acid magmas, that is, with an increasing SiO_2 content there is an exceptional decrease in the Ca:Al ratio

of the magma. A number of explanations have been proposed for these trends, including secondary alteration, vapour phase transfer, assimilation, crustal remelting, hydrous melting of the upper mantle and the fractional crystallisation of a variety of phases. We demonstrate here that corundum-normative magmas can be produced by the fractional crystallisation of a hornblende amphibole from calc-alkaline magma under moderate water pressure.

A large proportion of granitoid rocks are corundum-normative¹ and Chayes² found that 18% of 1,775 andesite analyses he compiled were also corundum-normative. By contrast, rocks of tholeiitic affinity are virtually always diopside-normative, even for the acid differentiates. This difference in normative mineralogy between calc-alkaline and tholeiitic suites has not been adequately explained.

Several calc-alkaline trends, which range from basaltic, through andesitic to dacitic and rhyolitic (gabbroic to adamellitic for intrusive suites) are plotted on Fig. 1, showing the transition from diopside-normative to corundum-normative. All the suites show a systematic trend of decreasing diopside content, eventually becoming corundum-normative with increasing SiO₂. The exact value of the SiO₂ content at which the rocks become corundum-normative varies considerably from about 56% for the Borrowdale volcanics³ to 70% for the Sierra Nevada batholith⁴ and the Cascades volcanics⁵. All the trends, however, appear to be subparallel.

Various models have been proposed for the production of corundum-normative magmas. They include:

Secondary alteration. The leaching of alkalis from pumice fragments after eruption could produce corundum-normative compositions⁶. It is, however, unlikely that 18.4% of all andesites² could be so affected. It is also remarkable that no tholeiitic suites contain leached compositions. The trends on Fig. 1 are regular. Leaching of alkalis would not be expected to produce as regular an alteration. Alkali leaching from intrusive rocks is very unlikely, and yet they show the same trend as volcanics. We do not accept alkali leaching as a general model.

Vapour phase transfer. Interaction of an aqueous vapour phase with magma could remove alkalis⁷. This could, however, only happen once the magma has become water saturated. The evidence does not support the idea that most calc-alkaline suites are water saturated. We do not accept it as the general mechanism for producing corundum-normative magmas.

Assimilation. Contamination of magma could produce a corundum-normative product, as has been demonstrated for the Taupo Volcanics, New Zealand⁸. But ⁸⁷Sr/⁸⁶Sr ratios do not indicate any significant contamination of two of the suites plotted in Fig. 1. The Cascades Volcanics⁹ and the Lesser Antilles Suite¹⁰ are not contaminated by crustal material. So we do not favour contamination as a satisfactory way of explaining all corundum-normative magmas.

Crustal remelting. Presnall and Bateman⁴ appealed to a process of fusion of andesitic crustal material to explain the chemical variation of the Sierra Nevada batholith (although they considered only the granitic component of the rocks). We note that partial melting of andesitic crust leaving an amphibole-rich residue could produce corundum-normative liquids, but corundum-normative magmas are observed where there is no significant continental crust, for instance, in the Lesser Antilles¹¹. Thus remelting of continental crust cannot be a generally applicable model.

Melting of hydrous peridotite. Kushiro and Yoder¹² have suggested that corundum-normative magmas could be produced by the melting of hydrous peridotite. That model does not, however, explain the continuous trend of compositions from basic to acidic. Furthermore, the model requires unrealistically high geothermal gradients for the mantle. The data were obtained under 10 kbar pressure¹², and Kushiro¹³ subsequently showed that partial melts from a hydrous peridotite were not corundum-normative at 20 kbar. Therefore, corundum-normative magmas would have to be formed at less than 20 kbar (60 km). Probable temperatures at this depth under

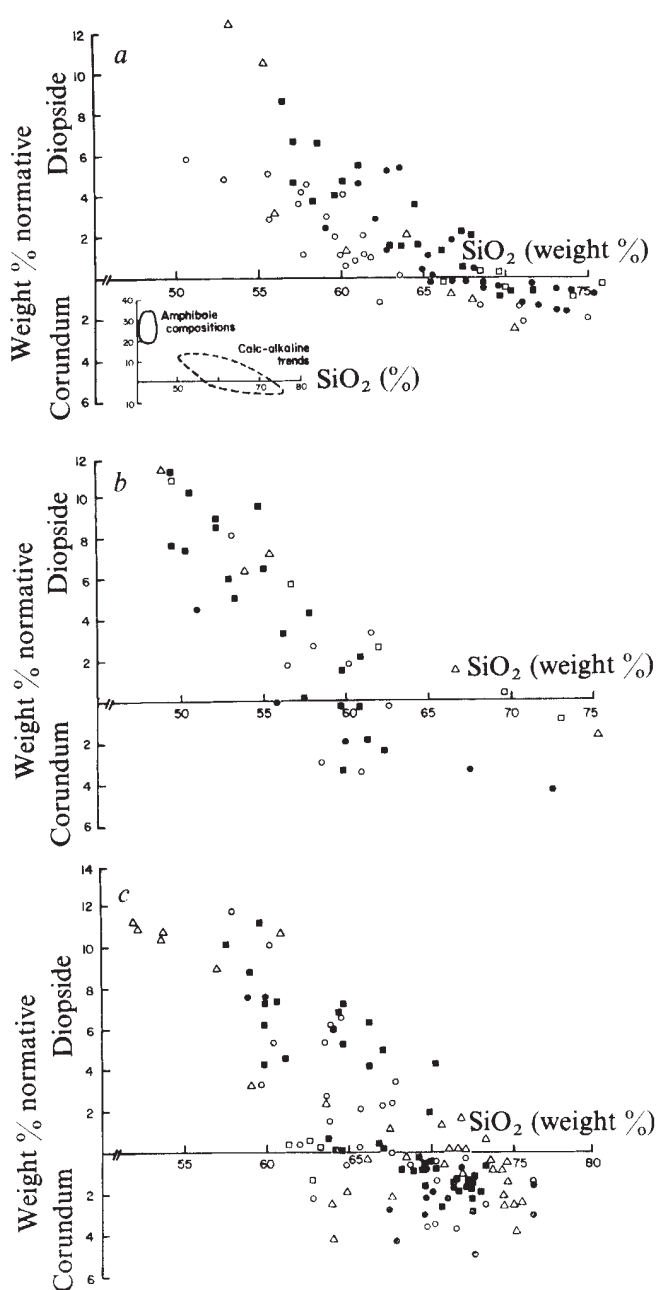


Fig. 1. Plots of normative diopside and normative corundum against SiO₂. *a*, Calc-alkaline intrusive magmas: ●, Sierra Nevada⁴; ■, Ben Nevis¹⁹; ○, Alaska²⁰ (Jurassic only); □, Namibia²¹; △, Garabal Hills²². *b*, Extrusive calc-alkaline magmas from various tectonic environments: ●, Borrowdale³; ■, Lorne²³ (basalt to andesite only); ○, St Kitts¹¹; □, Cascades⁵; △, Japan¹⁴. *c*, Lower Palaeozoic Appalachian rocks from Newfoundland²⁴: ●, Middle Ridge; ■, Holyrood; ○, Middle Brook; □, Deadman's Bay; △, Mount Peyton. (Inset on *a*: range of amphibole compositions synthesised from basic to andesitic compositions (see ref. 17) compared with the trends of the intrusive and extrusive calc-alkaline magmas.)

both continental and oceanic crust are too low to partially melt hydrous peridotite.

Fractional crystallisation. Kushiro and Yoder¹² also proposed that the fractionation of clinopyroxene could produce a trend from diopside-normative to corundum-normative. The clinopyroxene they synthesised would, however, not produce the trends observed in Fig. 1.

Kuno¹⁴ has suggested that the fractionation of magnetite-rich gabbro could produce the calc-alkaline trend. But there is a thermal divide at low pressure in the plane anorthite-forsterite-quartz¹⁵ such that crystallisation to produce a corundum-normative residue from diopside-normative liquids is not

possible. This is supported by Fitton's finding³ that clinopyroxene is never observed in corundum-normative compositions. So we do not believe that low-pressure crystallisation can produce corundum-normative liquids.

Green and Ringwood¹⁶ have suggested that garnet pyroxenite fractionation could produce the calc-alkaline trend, but they showed that the liquidus temperatures increased for compositions more siliceous than 61% SiO₂. In Fig. 1 the trends continue from 50% to over 70% SiO₂.

A fourth possible fractionation model for the genesis of the calc-alkaline suite has been suggested by Cawthorn and O'Hara¹⁷. They have suggested that amphibole fractionation may produce the observed chemical variation. We have plotted in the inset of Fig. 1 various amphibole compositions which have been synthesised from basic and intermediate compositions under 5–15 kbar pressure (see ref. 17). These plot on the back projection of the calc-alkaline trends and we suggest the crystallisation of such amphiboles explains these trends satisfactorily. Slight variations in slope of the trends in Fig. 1, may be attributable to different amphibole compositions crystallising at different pressures, or possibly to the coprecipitation of amphibole and plagioclase, which may become important in the most silicic compositions¹⁷.

Pinwinski and Wyllie¹⁸ have melted a series of corundum-normative granitoid rocks and shown that hornblende is the liquidus phase for most compositions. Thus, amphibole will crystallise from corundum-normative magmas and is capable of producing the characteristic trend of calc-alkaline magmas.

We therefore suggest that amphibole fractionation is the most plausible single model to explain all corundum-normative magmas (although the other models have a limited applicability).

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Argon isotopic evolution of upper mantle

BROWN *et al.*¹ have treated the problem of the history of the argon degassing of the Earth, and claim to demonstrate that the ⁴⁰Ar/³⁶Ar ratio in the mantle has increased from ~133, 770 Myr ago, to a value that "is at present not greater than the atmospheric ratio (295.5) and is probably a little lower". Their Table 1 and Fig. 1 show the results of K–Ar isochron analyses of several suites of "mantle-derived igneous" samples. We have been unable to reproduce in detail their results from the references they cite, and conclude that the apparent trend in their Fig. 1 is an artefact of data selection and that the source of the

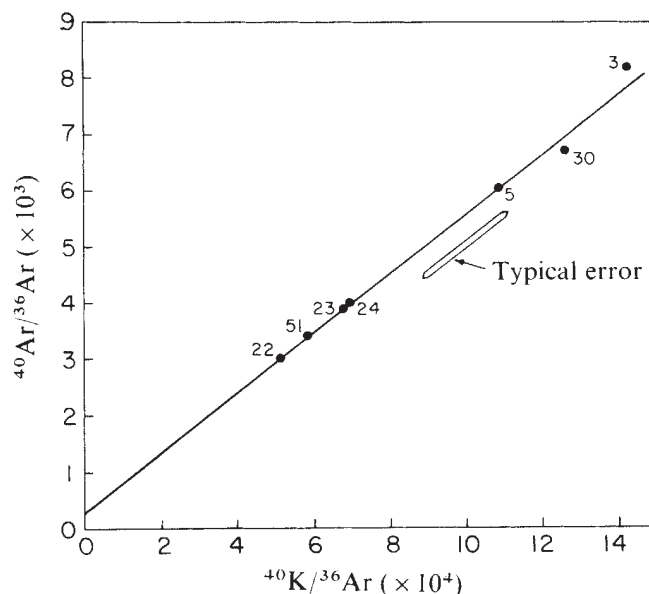


Fig. 1 A K–Ar isochron plot of group IIIA dyke data². The slope and intercept shown are from a regression line fitted to points 5, 22, 23, 24 and 51 (see text), which gives slope = 0.05274 ± 0.00052 ; an age of 736.7 ± 6.0 Myr; intercept ratio = 298 ± 34 .

observed argon has been misidentified. We think the ⁴⁰Ar/³⁶Ar ratios in the solid earth and the atmosphere have probably increased with time. Theoretical considerations and the available literature both indicate, however, that the ⁴⁰Ar/³⁶Ar ratio in the solid earth is much higher than the ⁴⁰Ar/³⁶Ar ratio in the atmosphere.

The apparent trend in Fig. 1 of Brown *et al.*¹ is mainly a function of the oldest datum, number 18, based on seven K–Ar analyses of the "group IIIA dike" samples² from the Beartooth mountains. These data are shown in Fig. 1 on a plot of ⁴⁰Ar/³⁶Ar against ⁴⁰K/³⁶Ar. A regression³ of all seven data points using a correlation coefficient of 0.9 yields an age of 745 ± 21 Myr and an ordinate intercept or

Fig. 2 A plot of (⁴⁰Ar/³⁶Ar)_i against age for the suite of samples chosen by Brown *et al.* The dashed line shows the present atmospheric ⁴⁰Ar/³⁶Ar ratio of 295.5. The error bars shown are one standard deviation (1σ) when the error is larger than the points. The small solid data points are from the calculations of this work. The open data points are those data from Brown *et al.* which differ significantly from ours. Points 15 and 16 of Brown *et al.* have been omitted because the data on which the points are based are not available in the scientific literature and points 2–7 have been omitted for reasons outlined in the text. The large solid data points shown for points 17 and 18 are the values shown in Table 1 and are our best estimates of the trapped gas in these two systems.

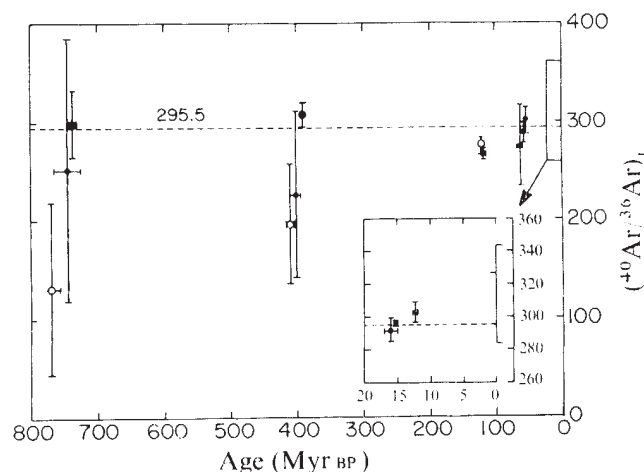


Table 1 'Measured' variation in $(^{40}\text{Ar}/^{36}\text{Ar})_i$ ratios

Point	$(^{40}\text{Ar}/^{36}\text{Ar})_i$		Age (Myr)		Ref.	Comments
	(This work)	(Brown <i>et al.</i>)	(This work)	(Brown <i>et al.</i>)		
1	283.8→342.4	282.9→294.8	Historic	Historic	10	Historic Basalts
2	295.1→326.0	292.1→320.4	Historic	Historic	9	'SP' single basalt flow
8	302.6±6.2	302±6	12.35±0.16	12.4±0.3	20	East Iceland Volcanics
9	296.4±0.6	295±3	15.20±0.05	15.3±0.2	21	Steens Mountain basalts
10	291.8±7.6	293±19	16.0±1.0	15.8±1.3	20	North-west Iceland Volcanics
11	302±11	289±21	51.7±21	53.1±2.5	22	Top of middle series Faroe Island basalts
12	274±42	263±21	61.7±5.6	64.8±3.1	22	Base of middle series Faroe Island basalts
13	287.9±9.9	287±9	55.2±1.2	55.4±1.2	22	Base of lower series Faroe Island basalts
14	266.4±6.2	275±8	117.0±3.8	119±4	5,6	Quartz diorite minerals
15	*	183±70	*	410±4	?	Etive Granites
16	*	224±37	*	415±4	?	Lorne Lavas
17	226±85 308±12	196±61	401.1±6.3 389.5±1.0	411±3	4 and pers. comm.	Argyll biotites
18	252±133 298±34	131±88	745±21 736.7±6.0	770±14	2	Dolerite dikes

*Data contained in a thesis not available to us.

Our results were obtained using a 1969 York³ regression program. The correlation between errors in the ordinate and abscissa of the isochron plots was assumed to be zero except as noted in the text. All of the listed errors are 1 s.d. (1 σ). The following values were used for the decay constants and abundance of ^{40}K : $\lambda_{\text{EC}} = 0.585 \times 10^{-10} \text{ yr}^{-1}$, $\lambda_{\beta} = 4.72 \times 10^{-10} \text{ yr}^{-1}$, $^{40}\text{K}/\text{K} = 1.19 \times 10^{-4} \text{ mol mol}^{-1}$.

initial $^{40}\text{Ar}/^{36}\text{Ar}$ ratio of 252 ± 133 . Five of the samples (numbers 5, 22, 23, 24, and 51), however, form a good linear array, and most of the error comes from the two most radiogenic samples (numbers 3 and 30). A regression of the five colinear data yields an age of $737.7 \pm 6.0 \text{ Myr}$, and an intercept of 298 ± 34 . We feel that this represents the best estimate of the age and intercept of this suite. Sample 3 contains slightly more radiogenic Sr than the other samples, which may indicate a small amount of crustal contamination. A regression of six samples (omitting number 3) yields an age of $715 \pm 12 \text{ Myr}$ and an intercept of 411 ± 72 . Brown *et al.*, however, omitted sample 30 and retained sample 3 in their regression, and we have found no objective reason for doing this.

Data from Argyll biotites (ref. 4 and a 1973 Oxford thesis (ref. 30 in Brown *et al.*)) are the source of data points 15, 16 and 17. Because the referenced thesis is unavailable we are unable to check points 15 and 16. We have, however, obtained the unpublished data (J. F. Brown, personal communication; R. J. Pankhurst, personal communication) included with the Argyll data for point 17. The regression of these data yields an age of $401.1 \pm 6.3 \text{ Myr}$ and an intercept of 226 ± 85 . Since the ^{36}Ar content in triplicate argon analyses of one of the samples (number 4 in ref. 4) shows almost as much variation, a factor of 1.6, as do all of the data combined, a factor of 1.9, it is clear, however, that these data define mixing lines, not isochrons. The triplicate analyses⁴ of sample 4 yield the best defined mixing line with an intercept of 308 ± 12 and an 'age' of $389.5 \pm 1.0 \text{ Myr}$. This mixing line is the best estimate of the trapped argon in this system. The system is clearly disturbed, however, since a regression of the data obtained by averaging the replicate analyses of each sample yields an age of 424.1 Myr and an impossible intercept of -98 ± 169 .

Data point 14 is taken from Hayatsu and Carmichael's⁵ treatment of data from mineral separates from a pyroxene quartz diorite in the Sierra Nevada of California⁶. The minerals show evidence of alteration⁶ and the hornblende datum lies above the line defined by the other minerals (see Fig. 5 of ref. 5). This set of minerals clearly represents a disturbed system. Point 14 also demonstrates graphically the danger of using someone else's K-Ar data for isochron plots. Brown *et al.* apparently assumed, as did Hayatsu and Carmichael⁵, that the column labelled " $^{40}\text{Ar}/^{36}\text{Ar}_{\text{total}}$ " in Table 2 of ref. 6 means radiogenic ^{40}Ar divided by atmospheric ^{40}Ar plus radiogenic ^{40}Ar . Kistler (personal com-

munication) indicates that " $^{40}\text{Ar}_{\text{total}}$ " contains a significant component of ^{40}Ar from the isotope dilution spike. After correcting for this component we obtain an age of $117.0 \pm 3.8 \text{ Myr}$ and an intercept of 266.4 ± 6.2 for the quartz diorite minerals—but the hornblende datum still plots off the line.

The rest of the data, points 1–13, have intercepts indistinguishable from the present atmospheric $^{40}\text{Ar}/^{36}\text{Ar}$ value. Some require comment, however. We calculate higher intercepts and lower ages for points 11 and 12 than do Brown *et al.* The amounts of ^{36}Ar in replicate analyses involved in points 8 and 10 show wide variations which indicate mixing lines.

Points 3–7 are from analyses of the Auckland volcanic field by McDougall *et al.*⁸. They do draw isochron diagrams but go on to conclude: "these linear arrays of data must be interpreted as indicating that the amount of excess Ar is nearly constant for a given volcano, and that the apparent isochrons are a result of this fact, together with the small variation of K in lavas from a single volcano" (p. 1517).

The data are from mixing lines, not isochrons, and do not belong in a compilation of 'initial' $^{40}\text{Ar}/^{36}\text{Ar}$ ratios. The clearest case is the data from the Rangitoto volcano. As McDougall *et al.*⁸ state, for Rangitoto "the radiocarbon, geological and botanical evidence unequivocally shows that it was active and was probably built in the last 1,000 years" (p. 1485). K-Ar data from Rangitoto define two apparent isochrons and two separate fits are made to one of these⁸, but the 'ages' which accompany these lines are ~500 and 200 times too large.

Point 2 is from work on the SP flow in the San Francisco volcanic field in Arizona⁹. The distribution of ^{36}Ar through the flow strongly suggests that the ^{36}Ar came from the atmosphere and not the mantle.

Point 1 is from work on modern surface volcanics¹⁰. Three samples (out of 27) had $^{40}\text{Ar}/^{36}\text{Ar}$ ratios less than the atmospheric value, but Krümmenacher¹⁰ demonstrated that these low values were due to mass fractionation effects on argon of atmospheric composition trapped in the lava and "not due to a mixture with primordial argon, that is depleted in ^{40}Ar ". Brown *et al.* report only three low $^{40}\text{Ar}/^{36}\text{Ar}$ data as their point 1. Baksi¹¹ has confirmed the basic observation that mass fractionation is often the dominant process in young volcanic rocks.

Our calculations indicate that Fig. 1 of Brown *et al.* should be replaced by Fig. 2 of this paper. The line shown

in their Fig. 1 is a physical impossibility since it extrapolates to -666 at $4,500$ Myr. We interpret our Fig. 2 as evidence that most, if not all, of the trapped argon in the samples shown in Table 1 and Fig. 2 is modern atmospheric argon which has been added to the samples during recent weathering and/or laboratory manipulation. There is an additional caveat. Shafiqullah and Damon¹² have recently discussed K-Ar isochrons and conclude that in geologically realistic situations K-Ar isochrons tend to be considerably more complicated than Rb-Sr diagrams, and should be used with great caution.

The best available samples of mantle gases are those trapped in the quenched rims of submarine basalts. These quenched rims of recent submarine pillow lavas contain argon with $^{40}\text{Ar}/^{36}\text{Ar}$ ratios higher than the present-day atmospheric ratio^{13,14}. It has been reported that the elemental ratios of He, Ne, Ar, Kr and Xe in some quenched rims are not atmospheric^{15,16}. This is interpreted as evidence that the quenched rims contain primordial rare gases from the mantle. In samples identified as containing mantle gases the $^{40}\text{Ar}/^{36}\text{Ar}$ ratio varies from 358 to $88,000$. Ozima and Kudo¹⁷ have argued that the present mantle $^{40}\text{Ar}/^{36}\text{Ar}$ ratio is approximately $2,000$. We believe that most workers would classify submarine basalts as 'mantle-derived igneous' rocks.

In summary, we can see no evidence for mantle argon with a $^{40}\text{Ar}/^{36}\text{Ar}$ ratio <295.5 . Data not considered by Brown *et al.* strongly indicate that the $^{40}\text{Ar}/^{36}\text{Ar}$ ratio in the mantle is ≥ 295.5 .

Brown *et al.* assume that "not more than 5% of crustal argon has been released to the atmosphere, and perhaps as little as 1%". They then calculate the amount of ^{40}Ar accumulated from K in the crust (39% of the Earth's total inventory of K in their model) in $4,500$ Myr, and release 5% of that ^{40}Ar in the atmosphere. Brown *et al.* implicitly assume that the entire crust has been in existence for $4,500$ Myr and has a current average K-Ar age of $4,412$ – $4,483$ Myr. This assumption violates all of the current theories of continental growth and is completely incompatible with what is known about continental age provinces¹⁹. While some argon derived from melting and recrystallisation might be trapped in the crust, the young average age of the continental crust, $\sim 1,100$ Myr, implies that most of the argon is degassed during uplift, weathering, metamorphism (radiogenic ^{40}Ar is found in natural gases²³), continental growth, and recycling in subduction zones. The fact that 5% of the crust is now sediments certainly does not mean that only 5% of crustal argon has been released to the atmosphere.

All of the detailed calculations of argon evolution of which we are aware yield mantle $^{40}\text{Ar}/^{36}\text{Ar}$ ratios higher than the corresponding atmospheric ratio throughout time. No matter what the initial partitioning of Ar was between the solid Earth and atmosphere, the atmosphere has no potassium. ^{40}Ar is produced only in the solid Earth. The $^{40}\text{Ar}/^{36}\text{Ar}$ ratio in the atmosphere can increase only by the degassing of the solid Earth. Degassing, however, removes both ^{40}Ar and ^{36}Ar from the solid Earth. While ^{40}Ar continues to be produced from the decay of ^{40}K , there is no mechanism which produces ^{36}Ar in the solid Earth. Therefore, the $^{40}\text{Ar}/^{36}\text{Ar}$ ratio in the solid Earth increases faster after a degassing event than it would have if the event had not occurred. Fractionating the potassium into a crust does not change the picture unless one is willing to postulate a differentiation process which removes the potassium while leaving the argon in the mantle.

In conclusion, we agree with the suggestion of Brown *et al.* that it may be possible to measure the time evolution of the $^{40}\text{Ar}/^{36}\text{Ar}$ ratio in the mantle using K-Ar isochron techniques. If such an evolutionary history can be obtained, it will provide an important boundary condition on petrogenetic and thermal models of the Earth. A similar evolu-

tionary history for the atmosphere may also be obtainable and would be equally important. We have shown, however, that Brown *et al.* have not measured the evolution of the mantle $^{40}\text{Ar}/^{36}\text{Ar}$ ratio. We predict that if and when such an evolutionary history of the mantle is reconstructed, it will bear no resemblance to that shown in Fig. 1 of Brown *et al.*

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WE thank Alexander and Schwartzman (AS)¹ for bringing to light a significant error in data point 18 (Table 1) of our paper². Because of a misinterpretation of a verbal communication from Mueller, we omitted sample 30 in our regression of the data from Baadsgaard and Mueller¹. We confirm that there is no reason for omitting this sample, and that the data indicate that the non-radiogenic argon present cannot be distinguished from atmospheric contamination. We concur with Alexander and Schwartzman, however, on little else, and consider as still valid the four principal points summarised at the end of our paper²: first, the initial $^{40}\text{Ar}/^{36}\text{Ar}$ ratios of mantle-derived igneous rocks have increased steadily over the past 800 Myr. Second, the present day $^{40}\text{Ar}/^{36}\text{Ar}$ ratios in the upper mantle and atmosphere are very similar. Third, the majority of argon at present in the atmosphere has been derived from the mantle rather than the crust, and fourth, initial $^{40}\text{Ar}/^{36}\text{Ar}$ ratios from igneous rocks are potentially more sensitive petrogenetic indicators than strontium or lead isotopes.

Alexander and Schwartzman are unable to reproduce similar ages in their Table 1 for two reasons: first, they consider all ^{36}Ar to be atmospheric contamination and second, they use a different half-life. We have used the half-life value recommended by Beckinsale and Gale⁴. Thus, as we approach the matter differently, we are likewise unable to reproduce in detail their Table 1 and Fig. 2.

With regard to point 17 of our paper, Argyll biotites, AS prefer to use the intercept of 3 analyses on one sample as the best estimate of the initial ratio. Since line blank is still a major problem in argon isochron analyses, and this sample was not blank corrected, the regression of these

three analyses will give an intercept corresponding to the blank $^{40}\text{Ar}/^{36}\text{Ar}$ ratio which is atmospheric in origin. Therefore it is not valid for AS to plot this point (17b) on their Fig. 2. Further an age of 389 ± 1 Myr, as suggested by this regression, is too young, since the samples predate, geologically, the granites and lavas of the region which are dated independently by Rb–Sr isochrons at 425 ± 5 Myr ($t_{1/2} = 5.00 \times 10^{10}$ yr).

Points 3 to 7 refer to McDougall *et al.*'s data³. We do not agree that the evidence for the young age of Rangitoto is unequivocal. The samples taken for the radiocarbon ages were covered by tuffs and a basalt flow which represent the latest activity on the volcano. The volcano could have been built up episodically over the past 0.5 Myr. Once again we are only suggesting an alternative interpretation.

A correction of Kistler's data⁵ to allow for the spike contribution yields a $^{40}\text{Ar}/^{36}\text{Ar}$ ratio even lower than we had thought. AS cite petrographic descriptions of the diorite as "evidence of alteration". The cited descriptions, however, indicate the quite pristine nature of these rocks⁷. The mantling of clinopyroxene by hornblende is a normal phase relationship developed during the crystallisation of diorites.

Krummenacher⁷ only reported three samples in his Appendix 2 as having a "true" $^{40}\text{Ar}/^{36}\text{Ar}$ ratio; these are the values we report as our point 1. The rest of his data are not tabulated or corrected in this fashion. Rather than ignoring the possibility of mass fractionation, we agree that it is an important factor which should not be forgotten when analysing young volcanic rocks. What we did say in our paper was that "the possibility of diffusion and fractionation of atmospheric argon into the cooling lava does not seem realistic to us". Krummenacher did not "demonstrate", he merely suggested that fractionation could be the cause of some low initial ratios.

For several of the groups of data AS have repeated the various explanations offered by the investigators (for example the quote by McDougall *et al.*³ regarding the Auckland data). We have not ignored the explanations given by others for apparently sub-atmospheric $^{40}\text{Ar}/^{36}\text{Ar}$ ratios, but have tried to offer a single alternative interpretation of the data, given the possibility that these low initial ratios do exist. This possibility has been considerably strengthened by the recent work of Mellor and Mussett⁸. The explanations of others, while perhaps equally valid, do not negate our interpretations. If one can explain low ratios in mantle derived rocks, any higher-than-atmospheric ratios present no problem; they may be explained by higher than average potassium concentration in the source region, extensive outgassing of the source region or xenolithic contamination of the rising magma. For this reason alone, one could justify the selection of low ratios for our model as they represent the closest approach to primordial argon from which mantle argon evolved. This justification is the same as that used in models of the mantle growth of $^{87}\text{Sr}/^{86}\text{Sr}$ ratios, even though the mechanics and complexities of the models are obviously different. It is not necessary to evolve elaborate mixing or fractionation models to explain low ratios in mantle-derived rocks, and these low ratios are no more 'anomalous' than the high ratios commonly thought of as containing 'excess argon'.

AS perceptively point out that the line shown in our Fig. 1 extrapolates to -666 at 4,500 Myr. Because of this, they claim the line is a physical impossibility, but such a conclusion is hardly valid. The line was intended to be nothing more than an indication of a first-order variation over the time indicated. The rate of growth of $^{40}\text{Ar}/^{36}\text{Ar}$ ratios in the mantle should not be linear, as it is strongly affected by the outgassing history. If Alexander and Schwartzman applied the same reasoning to their own Fig. 2, they might conclude that the atmospheric $^{40}\text{Ar}/^{36}\text{Ar}$ ratio 4,500 Myr ago was 295.5.

They make reference to Shafiquallah and Damon's⁹ caution against using argon isochrons; this is all very well if they concur with the main postulate of that paper: all ^{36}Ar measured in an analysis comes from the atmosphere. This postulate was discussed in our paper and rejected in favour of the assumption that a substantial proportion of the ^{36}Ar measured came from the sample itself. In their very next paragraph AS take the opposite stance and claim that the $^{40}\text{Ar}/^{36}\text{Ar}$ ratios measured in ocean floor pillow basalts represent the $^{40}\text{Ar}/^{36}\text{Ar}$ ratio of the mantle source region.

Perhaps we should have explained more fully our estimate of the amount of ^{40}Ar in the Earth's crust. For the sake of brevity and not anticipating such naïveté from interested readers we did not mention that the value of 4,500 Myr was selected because it is a defensible limiting condition and gives a maximum crustal ^{40}Ar value. We would be quite content with a smaller value because it is our contention that the crustal contribution to the atmosphere has been minor relative to the mantle contribution.

AS's statement that "the young average age of the continental crust, about 1,100 Myr, implies that most of the argon is degassed during uplift, weathering, metamorphism (radiogenic ^{40}Ar is found in natural gases), continental growth, and recycling in subduction zones" is fraught with fallacies.

Any such implication would require a comparison of the real age of all crustal material with the respective K–Ar ages. Such a comparison cannot be made, however, for the relevant K–Ar data are minuscule. The only relevant data are from unmetamorphosed rocks, where, in fact, the K–Ar ages are in good agreement with the real ages. The K–Ar data available for the vast metamorphic terranes of the crust are almost entirely from high potassium mineral phases, such as biotite. These minerals are hardly representative of continental crust. There are so little data from metamorphic rocks on the low-potassium phases (for example, plagioclase, pyroxene, and amphiboles), where 'excess argon' is common, that it is premature to attempt to evaluate the average K–Ar age of the continental crust.

Argon is not degassed during uplift. We are startled that Alexander and Schwartzman think otherwise. The evidence is too voluminous and familiar to geologists to cite here, but a large number of studies have shown that uplift results in argon retention.

Some argon is released to the atmosphere by weathering, but most of this would be taken into account in our consideration of sedimentary rocks. In fact it is possible that the retention of Ar by detrital material has led us to overestimate the significance of this surface process.

Metamorphism takes place at depth and the argon becomes mobile, at least on the whole-rock scale, but it cannot pass upwards through the cold capping rocks, except, perhaps along major fissures. If this were not the case, then the identification of excess argon in low potassium phases in metamorphic rocks would be extremely rare.

The fact that "radiogenic ^{40}Ar is found in natural gases" has no bearing on this matter. Alexander and Schwartzman refer to work done by Moorbath *et al.* in Iceland. It is perplexing to wonder what relationship they think this has to outgassing and metamorphism of continental crust.

There is absolutely no evidence that argon is outgassed during "continental growth and recycling in subduction zones". On the contrary, isotopic studies indicate the insignificant role of older continental material in the generation of new continental crust and also that there is either no recycling, or it is a process which has escaped detection.

One of the major differences between our models and the thesis of Alexander and Schwartzman is that they consider the whole Earth as being equally capable of degassing,

whereas we consider the upper mantle and the crust as separate systems with only the upper mantle having the capability for major outgassing through volcanism.

We believe that low initial $^{40}\text{Ar}/^{36}\text{Ar}$ ratios do exist in mantle-derived rocks and that these ratios reflect a mantle, or region of the mantle, which had a $^{40}\text{Ar}/^{36}\text{Ar}$ ratio of <295.5 when the rocks formed.

If the mantle does contain a source of argon which, not long ago, had a $^{40}\text{Ar}/^{36}\text{Ar}$ ratio less than the present-day atmospheric value, then it is relatively easy to explain any higher initial ratios observed. The real difficulty is encountered when one attempts to explain the derivation of low ratios from a mantle with high $^{40}\text{Ar}/^{36}\text{Ar}$ ratios. Alexander and Schwartzman recognise this difficulty, but can circumvent it by not accepting the existence of initial $^{40}\text{Ar}/^{36}\text{Ar}$ ratios less than the present-day atmosphere. Our interpretation has its own difficulties, but none so severe as indicated by AS. We have tried to account for less than atmospheric $^{40}\text{Ar}/^{36}\text{Ar}$ ratios in the mantle. The model promoted by Alexander and Schwartzman cannot account for, and in fact denies, the possibility of such ratios. It must distress AS somewhat to know that $^{40}\text{Ar}/^{36}\text{Ar}$ ratios significantly less than atmospheric have been directly measured from the Golovnin volcano (Kurile Islands) on two separate occasions by different researchers^{10,11}. Also there is now an example of an extremely low $^{40}\text{Ar}/^{36}\text{Ar}$ initial ratio in the 625-Myr-old Franklin diabase dykes¹², and data from the 60-Myr Antrim basalts⁸ indicate lower than atmospheric $^{40}\text{Ar}/^{36}\text{Ar}$ ratios in agreement with our model.

The real contention thus lies in whether or not mantle-derived rocks can contain $^{40}\text{Ar}/^{36}\text{Ar}$ ratios of less than that of the present-day atmosphere. Future research will resolve this contention.

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Temperature dependence of the anomalous specific heat of glasses and amorphous solids

THE low temperature specific heat of glasses and amorphous solids is characterised by an anomalous linear variation with temperature^{1–6}. Of the proposed theoretical explanations (reviewed by Leadbetter⁷), the most favoured attributes this anomaly to localised two-level systems^{8,9} or to low frequency vibrations of single atoms or molecules trapped in cavities^{10,11}. It is difficult, however, to conceive of a level-splitting mechanism that would be sufficiently general to explain the apparent universality of the anomaly. Moreover, several impurities that could have produced two-level systems have now been eliminated as possible candidates^{12,13}. An explanation based on low frequency vibrations of atoms or molecules in cavities rests on two *ad hoc* assumptions about the size and distribution of the cavities, and Stephens *et al.*⁵ argue that to account for the

experimental results, these assumptions seem to be physically implausible.

Experimental evidence implies that the amorphous state may be thought of simply as the crystalline state saturated with the most favoured dislocations¹⁴. This accounts for the densities of the crystalline and the amorphous state being very similar. Various aspects of the mechanical behaviour of metallic glasses have been discussed fruitfully in terms of dislocations and their movements^{15–19}, and we show here that this also accounts for the enhanced specific heat of glasses and for their anomalous linear temperature dependence.

Crystal defects can affect the specific heat of a solid by changes in the distribution of vibrational frequencies²⁰. Further, extended defects or dislocations can produce low frequency vibrations which are effective at low temperatures. At sufficiently low temperatures ($\sim 1\text{ K}$) it is not unreasonable to expect that dislocations will be pinned either by impurities or at their crossing points. Granato²⁰ analysed the pinned-dislocation contribution to the specific heat per mole, C_v , and found that at low temperature

$$C_v = \frac{\rho \pi^2 \Lambda a^2 N k}{3 Z \theta} T \quad (1)$$

where $\rho = v_o/C$ (v_o is the velocity of sound in the perfect lattice and C is given by the relation $C = (G/\rho)^{1/2}$ where G is the shear modulus and ρ the density), Λ is the dislocation density, a the lattice constant, Z the number of atoms per unit cell, N the number of atoms per mole, and θ the Debye temperature. Thus, for a given solid, the contribution of the vibrations of pinned dislocations to the specific heat is of the observed form. It is interesting to note that equation (1) depends on dislocations only through their density, and that the distribution of dislocation lengths is irrelevant. One also notes that neither the Burgers vector nor its distribution enters the expression. At $\sim 1\text{ K}$ the dislocation contribution to the specific heat may be of the same order of magnitude as that of the lattice. For heavily cold-worked aluminium at 1 K this contribution was found to be 20% of the total specific heat and a larger fraction at lower temperatures²⁰.

In seeking to obtain quantitative corroboration of these ideas one must contend with the fact that experimental data for glasses invariably refer to multi-element compositions, so average values must be inserted in equation (1). Golding, Bagley and Hsu²¹ find that for $\text{Pd}_{78}\text{Si}_{16}\text{Cu}_6$ the anomalous linear region gives $C_v/T = 1.3 \times 10^4 \text{ erg mol}^{-1} \text{ K}^{-2}$. Using the weighted averages $a = 3.8 \times 10^{-8} \text{ cm}$, $\rho = 10.3 \text{ g cm}^{-3}$, $G = 8 \times 10^{11} \text{ dyne cm}^{-2}$, $\theta \approx 344 \text{ K}$, $Z = 4$, and noting (with Granato²⁰) that $v_o = ak\theta/\pi\hbar$, one finds from equation (1) that $C_v/T = 5.6 \times 10^{-10} \Lambda \text{ erg mole}^{-1} \text{ K}^{-2}$. The saturation condition corresponds to the situation in which adjacent dislocation cores are contiguous, which implies that $\Lambda = (r_c^2\sqrt{3})/2$, where r_c is the core radius. For a typical metal, such as copper, it has been found that the core radius is ~ 3.6 Burgers vectors²². The most likely shear dislocation in close-packed structures is the Shockley partial with a Burgers vector of $0.41a$. Thus saturation corresponds to $\Lambda = 9.2 \times 10^{13} \text{ cm}^{-2}$, giving finally $C_v/T = 5.2 \times 10^4 \text{ erg mol}^{-1} \text{ K}^{-2}$. In view of the difficulty involved with using average values the agreement with the result of Golding *et al.* is quite good.

Because core radii are invariably a few $\text{\AA}$, the saturation dislocation density is roughly constant so it is not surprising that the anomalous specific heat contribution for different substances does not vary greatly^{2,6}. Further, the scattering of phonons by dislocations implies that the thermal conductivity, if this type of scattering is dominant, would be $\propto T^2$. It is experimentally observed that the temperature exponent of the thermal conductivity for non-crystalline solids varies between 1.8 and $2.0^{2,6,23}$. (It should be noted that although Lasjaunias *et al.*²³ found that the thermal conductivity of vitreous B_2O_3 between 0.1 and 1.3 K varies as $T^{1.9}$ the excess specific heat contribution seemed to vary as $T^{1.45}$ between 60 mK and 0.6 K. The reason for this behaviour is not clear to us.) In the limit as $T \rightarrow 0$ equation (1)

is invalid, and Granato²⁰ gives an expression

$$C_v = \frac{\Lambda}{L} \frac{a^3}{Z} Nk \left(\frac{\hbar \omega_0}{kT} \right)^2 \exp(-\hbar \omega_0/kT) \quad (2)$$

which goes exponentially to zero at very low temperatures (ω_0 corresponding to the lowest vibrational mode). This indicates a method of testing these ideas.

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Cementite structure for iron sulphide, Fe₃S

IN our studies on the growth of iron sulphides in an inert gas, we synthesised a new crystal form of iron sulphide having a cementite-type structure. The method was the following: a fine powder of synthetic troilite (Fe_{1.0}S) was flash evaporated in 99.99% pure argon gas at 100 mmHg, and passed into a vacuum chamber initially at a pressure of 10⁻⁶ mmHg. A thin film was obtained on some water-cooled microgrids 10 mm from the evaporation source (1,900 °C) and was examined in a Hitachi HU-11D electron microscope equipped with the model HXA-1 electron probe microanalyser (with mica as dispersing crystal).

The intensities and lattice spacings as observed and calculated are listed in Table 1. The pattern can be indexed on the basis of an orthorhombic unit cell with $a=4.56$ Å, $b=5.09$ Å, $c=6.70$ Å.

Cementite¹, Fe₃C, space group Pbnm, has an orthorhombic unit cell with $a=4.523$ Å, $b=5.088$ Å and $c=6.743$ Å. On the other hand, vanadium subsulphide² β-V₃S is known to have a tetragonal unit cell (P4₂/nbc) with $a=9.381$ Å and $c=4.663$ Å. From the better agreement with the lattice constants of the former, the new crystal probably has the cementite structure. The electron diffraction data for the iron sulphide yield no further information, since there are too many overlapping and coinciding peaks.

The chemical composition of the thin film was established roughly by X-ray emission spectra, using 2C-type pyrrhotite film (FeS) as a standard: a film was prepared by the flash evaporation of the same starting material (Fe_{1.0}S) in argon at 35 mmHg, and its chemical composition was determined by the presence of the $d(100)=5.16$ Å line of the 2C-type superstructure. The intensity measurements of the Kα lines of Fe and S reveal that the new phase is very much richer in iron than sulphur, and the composition falls between Fe_{2.2}S and Fe_{3.8}S.

Table 1 Electron diffraction data for the new cementite-type iron sulphide

hkl	d _{obs}	d _{calc}	I _{obs}	hkl	d _{obs}	d _{calc}	I _{obs}	hkl	d _{obs}	d _{calc}	I _{obs}
001		6.70		203		1.60		232		1.26	
010		5.09		014	1.59	1.59	16	303	1.25	1.26	8
100		4.56		130		1.59		041		1.25	
011		4.05		123		1.57		115		1.25	
101		3.77		104		1.57		140		1.23	
110	3.39	3.40	10	131		1.55		313	1.22	1.22	19
002		3.35		213	1.53	1.52	9	322		1.22	
111		3.03		300		1.52		141		1.21	
012		2.80		222		1.51		224		1.19	
102		2.70		032	1.50	1.51	1	034		1.19	
020	2.53	2.54	5	114		1.50		042		1.19	
112		2.38		301		1.48		025		1.19	
021	2.39	2.38	8	310		1.46		233	1.17	1.16	10
200	2.28	2.28	15	132		1.44		205		1.16	
003		2.23		311		1.42		134		1.15	
120	2.22	2.22	17	024	1.40	1.40	3	142		1.15	
201		2.16		302		1.38		125		1.15	
121	2.11	2.11	36	230		1.36		400	1.14	1.14	25
210	2.08	2.08	53	033		1.35		330		1.13	
013		2.04		223		1.35		215		1.13	
022	2.04	2.03	100	204		1.35		323		1.13	
103		2.00		005	1.34	1.34	12	304		1.13	
211	1.99	1.99	39	124		1.34		401		1.12	
202		1.88		312		1.34		006		1.12	
113	1.87	1.87	25	231		1.33		331		1.12	
122	1.84	1.85	18	320		1.30		410		1.11	
212	1.76	1.77	15	214		1.30		240	1.11	1.11	21
030		1.70		015	1.29	1.30	8	043		1.11	
220	1.69	1.70	12	133		1.30		314		1.10	
023		1.68		105		1.29		411		1.10	
004		1.67		321		1.28		241		1.10	
221		1.65		040		1.27		016		1.09	
031	1.64	1.64	4					106		1.08	

$$a = 4.56 \text{ Å}, b = 5.09 \text{ Å}, c = 6.70 \text{ Å}.$$

It is postulated that the film-like deposits form at 100 mmHg because of the high density of vapour in the narrow condensation region which comprises different supersaturation zones. This is confirmed by the fact that thin films composed of 2C-type pyrrhotite (FeS), marcasite and pyrite (FeS_2) were obtained near the evaporation source except for a few regions where the iron-rich sulphide was the single phase.

There are two restrictions on the conditions of film formation of the iron sulphide in this experiment: first, the only position at which the thin film could be obtained was at a distance of 7–15 mm from the evaporation source. Further away, the subsequent growth leads to the formation of iron sulphide smoke particles, until they aggregate into a chain of composition Fe_{1-x}S . Second, at lower pressures, 5 and 35 mmHg, the thin film obtained at the same position was not the cementite-type iron sulphide, but only 2C-type pyrrhotite used above as the standard sample. Further details will be published elsewhere.

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Growth rates of freely falling ice crystals

THE only available set of laboratory measurements of mass growth rates of freely falling ice crystals, as a function of both time and temperature, used growth times < 50 s (ref. 1). We report here results of laboratory measurements on freely falling ice crystals (average mass, $\bar{m}$) grown in simulated cloud conditions, as a function of temperature for growth times of 60 ± 3 and 100 ± 5 s, and from these we have calculated the corresponding mass growth rates.

We wished to simulate the growth processes occurring in natural clouds, and therefore the measured ice crystals were produced in, and then grew while falling freely

Fig. 1 Average mass of freely falling ice crystals as a function of temperature for growth times of 1, 60 ± 3 s and 2, 100 ± 5 s.

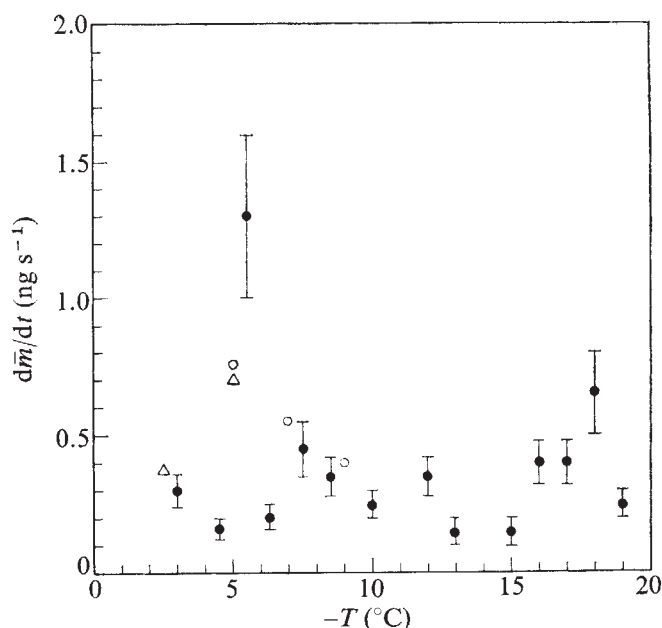
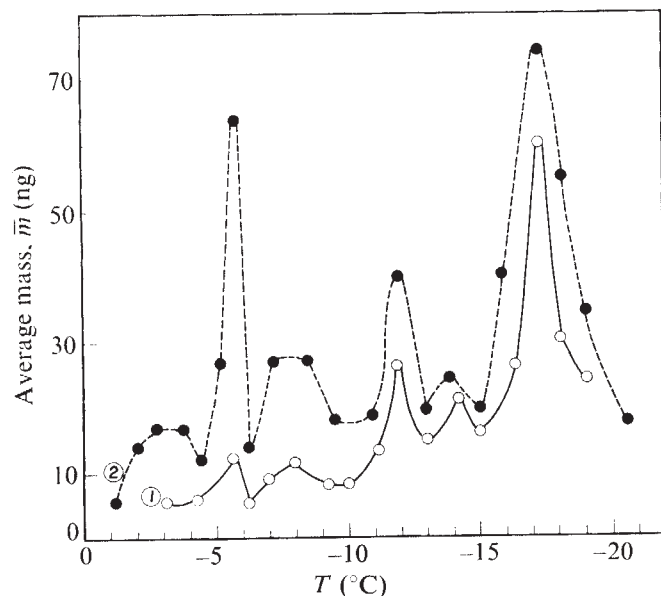


Fig. 2 Average mass growth rate of freely falling ice crystals as a function of temperature in the time range 60–100 s, calculated from: this work (●); Mason⁹ (△) and Ryan *et al.*¹⁰ (○)

through, fogs of supercooled water droplets with size spectra and concentrations similar to those of many rain clouds. The ratio of droplets to ice crystals was always > 8 : 1.

Ice growth was initiated by the rotation of a metal rod, cooled with liquid air, which locally seeded the supercooled fog with tiny ice crystals. The settling ice crystals were sampled by collecting them on a cooled glass slide coated by a layer of highly viscous silicone oil (DC 200/1,000). Each of the samples comprised ~ 150 crystals.

The average mass values of the samples were calculated from the measured diameters of the water droplets resulting from melting the collected ice crystals. Details of the experimental system and procedure are described elsewhere².

Figure 1 shows the temperature-dependent average mass of ice crystals, sampled at 60 ± 3 and 100 ± 5 s after seeding, and Fig. 2 shows their calculated temperature-dependent average mass growth rate in the time range 60–100 s.

It is obvious from Fig. 2 that, first, there are peaks in the growth rate at ~ -5.5 and -18 °C, as have been found previously^{1,3,4}; second, the maximum at about -5.5 °C is similar to the behaviour of the basal linear growth rate⁵, basal mean migration distance⁶ and basal step propagation velocity^{7,8}; and third, the few growth rates for the range 60–100 s, calculated from experiments by Mason⁹ and Ryan *et al.*¹⁰ are in good agreement with our results.

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'Braille' reading by a blind volunteer by visual cortex stimulation

THE possibility of developing functional visual prostheses was supported by the pioneering case of Brindley and Lewin^{1,2} and confirmed by our subsequent report on two other blind patients³.

Although much work remains to be done on engineering design^{4,5} and on minimising the effects of continuous chronic electrical stimulation of the brain^{6,7}, the most fundamental question remains the demonstration of useful information transfer by cortical stimulation.

We therefore report preliminary experiments in which a blind volunteer was, without practice, able to read 'braille' transmitted by cortical phosphenes at 30 letters min⁻¹ (much faster than he could read tactile braille). This patient was also able to use a TV camera to detect horizontal and vertical lines. These experiments with dynamically changing pattern presentation are the first demonstration of potentially useful information transfer by cortical stimulation.

Similar attempts to transmit 'cortical braille' to Brindley's second patient⁸ were hampered by large, anomalous phosphenes which limited reading to 8.5 characters min⁻¹, much slower than the patient could read tactile braille. It should, however, be emphasised that as yet we regard 'cortical braille' primarily as a technique to begin investigation of dynamic pattern presentation, rather than as a basis for clinically useful prostheses.

Our previous experiments^{3,9} used temporary electrode arrays removed a few days postoperatively, but the time limitations of this approach are a serious drawback. Consequently, we designed a chronic implant (Fig. 1) by modifying our ribbon cable electrodes to connect with a special percutaneous pedestal¹⁰ developed in the course of our parallel programme on auditory prostheses.

The volunteer, a 33-yr-old man, was blinded ten years ago by gunshot. He had been associated with the project for several years and his fully informed consent had been obtained³. Surgical techniques and postoperative instrumentation were similar to those described earlier³, with the addition of a camera based on a 100×100 charge-coupled phototransistor array interfaced with the computer system.

Most stimulation has been conducted with 0.1–1.0-s trains of constant current, symmetrical, biphasic square pulses, coupled through 0.47 μ F series capacitors, typically of zero to peak amplitude 0.5–3.0 mA, of 0.25/0.25 ms duration (–/+), repeated at 50 Hz. All simultaneous stimulation of multiple electrodes involved individual amplitude adjustments to a constant multiple (typically 1.25) of threshold, and all trains were interlaced typically with 1.0-ms intervals between pulses delivered to different electrodes in numerical order.

Sixty of the 64 electrodes have continuous connecting wires. All produce point phosphenes (some are multiples) at thresholds ranging from 0.8–4.0 mA zero to peak (1.7 mA average with a median of 1.4). These thresholds fluctuated during the postoperative consolidation period, stabilising during the fourth week. They do not seem affected by the limited, intermittent, stimulation involved in our experiments to date. As expected⁹ there are no significant differences in phosphene appearance or thresholds based on electrode area.

Electrode 'impedances'⁶ are complex and nonlinear. For 0.25/0.25 ms duration (–/+) pulses, 5.2–21.5 V peak to peak (average 8.5; median 8.4) are necessary to force 3.0 mA peak to peak. There is no clear correlation of 'impedance' with either threshold or electrode area.

A detailed map (Fig. 2) of the relative position of each phosphene in the visual field was prepared under computer control³ with adjustments for absolute distances between

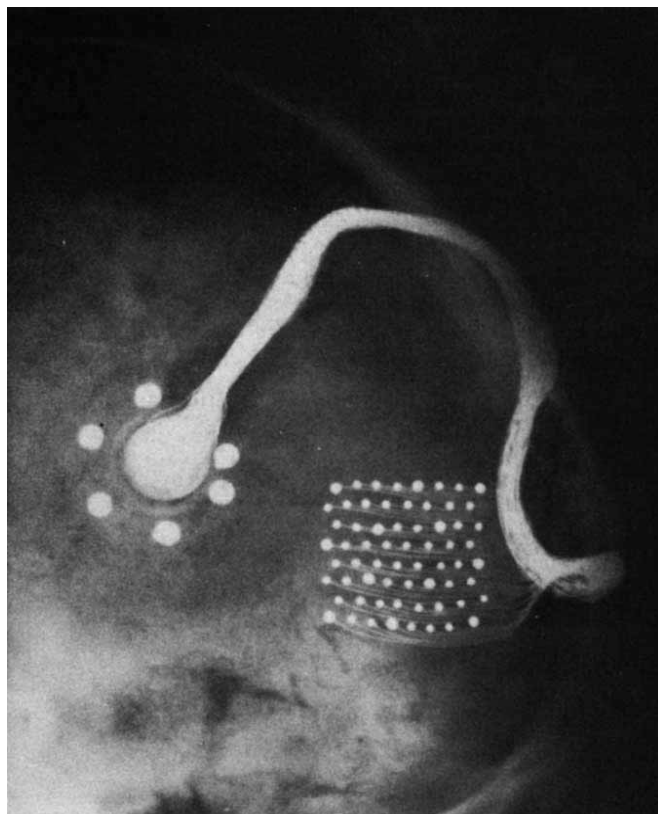


Fig. 1 Array of 64 platinum electrodes in a Teflon matrix. They are hexagonally packed on nominal 3-mm centres, in the subdural space on the right medial occipital cortex. 12 electrodes are 2 mm² in area and the remainder 1 mm². In this X ray they are numbered in horizontal rows (number 1 in the upper right, number 8 in the upper left, number 57 in the lower right, and number 64 in the lower left). The integral platinum connecting wires are individually insulated with Teflon and form a small cable which passes through the skull and folds beneath temporo-occipital periosteum. The wires terminate in a special high density connector inserted in a percutaneous Pyrolite carbon pedestal, attached to the cranial vault with special bone screws.

phosphenes based on a manually prepared map.

As previously reported³ simple letters and patterns can be recognised easily, although this is hampered by interactions which often occur between phosphenes which are neighbours in visual space (regardless of the position of the corresponding electrodes in the array). The number and spatial distribution of phosphenes is inadequate for presentation of 26 ordinary letters. Consequently, as indicated in Fig. 2, six non-interacting phosphenes were selected to form a 'braille cell'.

Using randomised alphabets, presented as 0.5-s trains of cortical stimulation at intervals controlled by the patient's response, he averaged 19 (17–20) correct responses out of 26 letters (73%) over an average period of 47 (44–56) s. Changing the method of presentation slightly so that each letter was represented by a 0.5-s train at regular 2.0-s intervals, the patient averaged 85% correct responses in three trials with similar random alphabets.

The results with 'cortical braille' contrast sharply with similar experiments using tactile braille scanned at the same rate. Random alphabets were punched on file cards to optimise tactile sensation, and the patient permitted to examine each letter for 2 s. A tone indicated a shift to the next letter, and in these conditions the patient averaged only 28% (23–35%) correct responses in each of three trials.

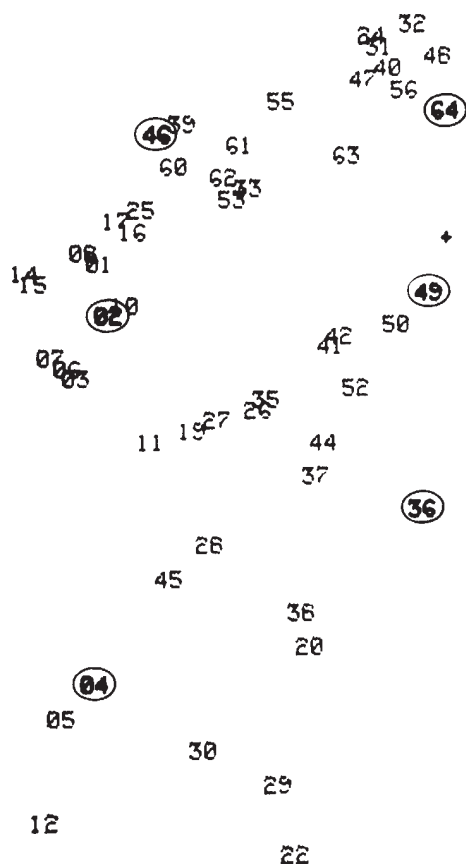
We then changed to sentence presentation using 'cortical braille' at a rate of 30 characters min⁻¹ (0.5 s trains delivered every 2 s, with a 4-s pause between words). The initial presentations (selected from a braille reading test) and responses were as follows: (1) "When the crow went into"

(54 s) read as "... crow went n to". (2) "He had a cat and ball" (52 s) read as "He his cat and ball". (In this case he actually spelled the word "has" properly out loud but called it "his".)

The patient who has a high-school education is a very poor braille reader, averaging about 1 word min⁻¹ on high school level, grade II (contracted) braille; consequently, he rarely uses tactile braille. He believes, and we concur, that he will become considerably faster at 'cortical braille' with practice, particularly when he can control the rate of presentation through a modified tablet now being interfaced with the computer. Preliminary experiments with kinaesthetic feedback were conducted using the IC camera interfaced with the computer system (0.5-s trains with a maximum of 8 electrodes per frame). Although complicated by phosphene interaction, the patient could distinguish horizontal and vertical strips of 1 inch wide white tape on a black background, and further experiments are now in progress.

Experiments with 'cortical braille' and the camera emphasise the need for interframe delays, particularly when using very short trains (for example, 125-ms trains repeated at 250-ms intervals). At frame rates faster than 4 s⁻¹, presentations tend to blur, regardless of the patient's ability to recognise and retain individual characters. From the patient's description, we believe that such blurring results from short duration phosphene persistence, compounded

Fig. 2 Cathode ray tube map of phosphene positions in visual space. The phosphene produced by electrode number 22 is about 22° from the fixation point marked by (+). As expected^{1-3,9} there are many discrepancies from the classical retinotopic map. For example, electrodes far from the occipital pole produce central phosphenes. Adjacent electrodes (for example numbers 30, 31 and 32) may produce widely spaced phosphenes, perhaps due to intervening fissures. Adjacent phosphenes, however, produced by distant electrodes (for example numbers 1 and 16) are more difficult to explain. The circled bold numbers indicate the 6 phosphenes forming the 'braille cell' (electrodes number 46, 2, 4, 64, 49 and 36).



by the intrinsic phosphene flicker. Further experiments on both of these phenomena are also in progress.

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Ethylene-induced volatile inhibitors causing soil fungistasis

THE phenomenon of soil fungistasis¹⁻³, a term introduced by Dobbs and Hinson⁴ to describe the failure of almost any fungus propagule to germinate in the upper layer of non-amended natural or cultivated soil, has been attributed to volatile inhibitors. Evidence implicating volatile unsaturated hydrocarbons was first obtained from the observation that when soil blocks are incubated in closed containers over either silver nitrate or mercuric perchlorate solutions, they exhibit reduced fungistasis activity against conidia of *Arthrotrichum oligospora*².

To test the hypothesis that ethylene is the main inhibitor involved in soil fungistasis³, nutrient-free water agar disks 2 mm thick (Special Agar Noble, Difco), carrying conidia of *A. oligospora* were incubated at 22 °C in atmospheres containing up to 50% (v/v) ethylene (Ethylene CP Calibration Mixture, Commonwealth Industrial Gases). Contrary to what should have been expected if ethylene had any fungistatic activity, conidia germination in all cases proceeded unrestricted, and equalled that of the control series in ethylene-free atmospheres. The use of nutrient-free agar renders unlikely the argument that results obtained in pure culture cannot be extrapolated to unsterilised soil because the fungistatic action can be overridden by nutrients⁵. This

becomes even more apparent in view of the following tests. Using a modification of the Romine and Baker soil emanation technique⁷, agar disks, similar to the ones used in the ethylene bioassays, were laid in disposable Petri dish halves (3.5 cm diameter). These were placed inverted on the surface of 100-g soil samples. A glass vial (10 ml), containing a folded piece of glass-fibre paper and either silver nitrate solution (2.5% w/v) or water, was included in the headspace of soil in each Petri dish. The soils, in this and subsequent experiments, were collected on the day of use and sieved to 0.2 cm while still moist. After 3 d incubation at 23 °C, the fungistatic activity of the agar disks was assessed by seeding, on their lower surface, conidia of either *Penicillium chrysogenum* or *P. italicum* and counting the percentage germination after 18 h further incubation in the same conditions. The experiment was repeated a number of times in triplicate using originally Irish (clay loam, pH 6.6, University College, Dublin) and subsequently Tasmanian (sandy clay loam, pH 5.5, Hobart area) soils. On all occasions the results were of comparable order and showed that the agar disks of the silver nitrate series were less fungistatic compared with controls. Typical results are presented in Table 1. Kouyeas, in the Agricultural College of Athens, using a similar experimental arrangement and four soils from southern Greece (pH 7.6–7.9), obtained comparable results with eight different fungal species⁸.

The fact that agar disks acquire fungistatic properties when exposed to volatile emanations from soil, but not when incubated in the presence of ethylene, leaves no doubt as to the ineffectiveness of the latter as a spore germination inhibitor. Examination of the electronic spectra of water condensates collected from soil headspace revealed, however, that ethylene-like substances were present in the soil gaseous emanations.

In these experiments, an all-glass cold finger condenser, with a small vial suspended from its lower end, was suspended over 500-g soil samples held in conical flasks (2 l). During the experiment the soil temperature was kept at 25 °C whereas that of the water circulating in the condenser was about 10 °C. An incubation period of about 24 h was usually sufficient for the collection of 5–10 ml water condensate in the receiver vial, beneath the condenser.

Water condensates from both Irish and Tasmanian soils had a greater A_{196} than distilled water. By adding dropwise a freshly prepared bromine water solution into the sample cell only, an absorption band emerged in the region of 165 nm, the intensity of which increased with further addition of bromine.

Trimethylamine or formic acid (1 mg l⁻¹) could account for the A_{196} , but neither showed any response to bromine treatment; and when they were neutralised both failed to inhibit spore germination. Water saturated with ethylene or solutions of other compounds containing an ethylene double bond, when treated with bromine, gave an absorption curve similar to the one that was given by soil water condensates under the same treatment (Fig. 1), indicating the presence of ethylene-like substances among the soil gaseous emanations.

Since ethylene itself has no inhibitory effect on spore germination, however, Smith's observation, that soil fungistasis is enhanced in the presence of ethylene^{5,9}, leads to the alternative hypothesis that ethylene might trigger or accelerate

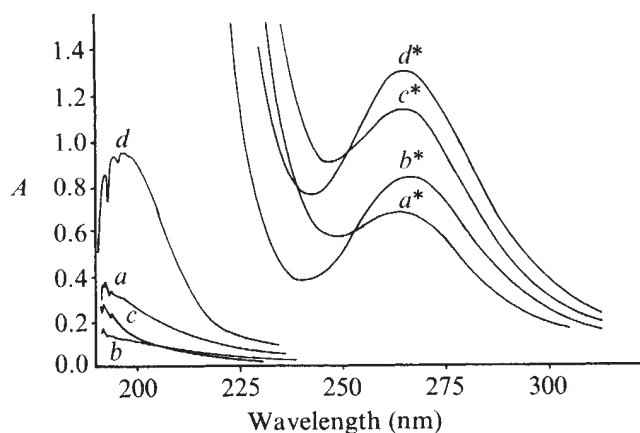


Fig. 1 Absorption spectra of water condensates of soil headspace (a), water saturated with ethylene (b), allyl alcohol (c) and acrylic acid (d). a*–d*, Same as above after treatment with bromine.

the process by which the inhibitor(s) is produced. Water condensates were collected, as described above, from soil samples (1 kg fresh weight) which had been pre-incubated for 3 d in atmospheres of air only or air supplemented with 1% ethylene. After collection, the water condensates were transferred to glass vials, which were sealed with rubber septa and placed in a 60 °C water bath. Gas samples (2 ml) were withdrawn from the headspace of each vial and analysed in a Pye Unicam 104 Gas Chromatograph with a 1.60 × 0.003 m column packed with 5% Carbowax 20M on Gas Chrom Q (80/100 mesh), 60 °C, N₂ carrier gas and H₂ flame ionisation. Condensates from the ethylene-treated soil yielded, apart from a peak attributable to ethylene, several others with poor resolution and one with retention time similar to allyl alcohol (approximately 5.5 min). Characteristically, no peak was registered when samples of water condensates from air-treated soil were used. Incubation of soil in presence of ethylene thus resulted in the production of other volatile substances; although they are awaiting identification and assessment of their significance, formation of allyl alcohol alone may account for the enhancement of fungistatic activity in soils treated with ethylene as the following microdiffusion germination tests indicate.

Although acrylic acid was not detected, it was included in the bioassays because of its close similarity to allyl alcohol, the fact that Shmuk (cited in ref. 10) had included this acid in a list of organic substances isolated from soil by numerous investigators, and its identification with the factor responsible for the antibacterial properties of phytoplankton and seaweeds^{11–14}.

Bioassays were carried out using a microdiffusion system designed to simulate the conditions of the spore germination tests on soil blocks² and those of the agar disk technique above. Films (1 mm thick) of nutrient-free agar carrying conidia of *A. oligospora* were incubated over solutions of either allyl alcohol or acrylic acid in containers similar to Conway's microdiffusion dishes (3.4 cm diameter, 2 cm deep; central compartment, 1.4 cm diameter, 1 cm deep). Aliquots (5 ml) of the compound under test were placed in the outer compartment of the dishes whereas the inner compartment received 1 ml silver nitrate solution (2% w/v) and a small pleated piece of glass-fibre paper to increase the absorbing capacity of the solution.

Table 2 shows that the diffusing vapours from both allyl alcohol and acrylic acid solutions were fungistatic and that the presence of silver nitrate resulted in a dramatic reduction of their inhibitory effect. The fact that allyl alcohol seems more effective (quantitatively) than acrylic acid

Table 1 Percentage spore germination on nutrient-free agar disks pre-exposed to volatile emanations from soil, in the presence or absence of silver nitrate solution

Soils	Test organisms	Silver nitrate solution	
		Present	Absent
Irish	<i>P. chrysogenum</i>	62 ± 5.0	44 ± 3.5
Tasmanian	<i>P. italicum</i>	80 ± 5.3	52 ± 6.2

Table 2 Percentage germination of *A. oligospora* conidia in micro-diffusion tests of allyl alcohol and acrylic acid in presence or absence of silver nitrate solution

Test compound	Silver nitrate solution	
	Present	Absent
Water (Control)	95 ± 2.1	96 ± 1.2
Allyl alcohol (4 p.p.m.)	46 ± 4.6	13 ± 0.8
Acrylic acid (1,000 p.p.m.)	77 ± 3.4	7 ± 2.0

should not be considered as evidence lessening the possible ecological significance of acrylic acid. The microdiffusion system used does not give a direct measure of the potency of the inhibitors but reflects the balance between the rate of diffusion and subsequent accumulation of the inhibitor in the agar films and the rapidity with which spores are germinating. It is, therefore, the relatively high speed with which the conidia of *A. oligospora* germinate and presumably, the low diffusion rate of vapours of acrylic acid, together with a moderate activity, that result in an underestimation of its effectiveness as a fungistatic agent. Thus, when agar films were immersed for a few minutes in acrylic acid solutions and used for germination tests, the effective dose for 50% inhibition was reduced to only 40 p.p.m.

Presence of nutrients counteract the soil fungistatic activity. Similarly, the inhibitory effect of both allyl alcohol and acrylic acid increases progressively with the decrease of the nutrient status of the spores. Note that both the above compounds, within a certain concentration range, depending on the fungus species and the level of nutrients, do not impair the viability of the spores for a considerable period of time (C.B., unpublished).

Brian *et al.*^{15,16} have studied the fungistatic activity of a number of ethylene-like compounds and indicated that fungistatic activity is related to the tendency of the substituent groups to withdraw electrons from the double bond^{14,15}. This proposition readily explains the contrast in activity between ethylene and allyl alcohol and acrylic acid. The activity of the latter is probably restrained because of the low permeation of cells by organic acids.

My results are in accord with Smith's observation that ethylene causes soil fungistasis⁵, except that the main inhibitor of spore germination is not ethylene but the ethylene-induced and tentatively identified allyl alcohol and perhaps other products. Smith's concept of the oxygen-ethylene cycle⁹ may, therefore, represent only part of a more complex system awaiting elaboration.

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Rhythmic oscillations of phytochrome and its pelletability in *Cucurbita pepo* L.

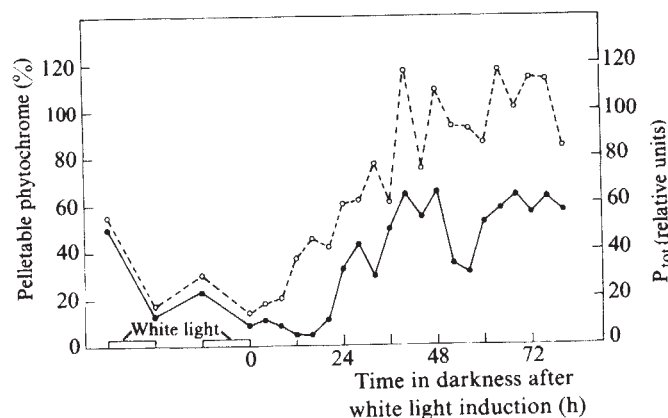
MANY reactions of plants (such as flower initiation, growth and chlorophyll synthesis) show a rhythmically changing sensitivity to light¹. According to Bünning's hypothesis, the photoperiodic reactions are determined by the interaction between an endogenous rhythm and phytochrome: both components are essential for a photoperiodic response. The endogenous rhythm determines the changing sensitivity of the plant to P_{tr} , the physiologically active form of phytochrome. These characteristic changes in the sensitivity of the system towards light are probably not attributable to different P_{tr} molecules. On the basis of the theory that the binding of P_{tr} to a membrane is the primary reaction of phytochrome, it has been suggested² that such sensitivity changes could be achieved by varying the interaction of phytochrome molecules with their receptor sites. We have investigated the association of phytochrome with pelletable structures^{3,4} and found that after a 12-h white light induction the total amount of phytochrome and its pelletability oscillate in the dark with frequency periods of 24, 12 and 6 h.

For routine preparations of particulate fractions, pre-chilled tissue—cotyledons or hooks of Squash (*Cucurbita pepo* L., cv. Black Beauty)—were chopped with a razor blade and homogenised with a mortar and pestle in extraction medium. In all experiments the medium-tissue ratio was 3:1 (v/w). The medium contained 0.25 M sucrose, 0.1 mM $MgCl_2$, 14 mM 2-mercaptoethanol, 25 mM N-morpholino-3-propanesulphonic acid and 3 mM EDTA, pH 8.0. The pH in the final homogenate was about 7.0. The homogenate was squeezed through nylon cloth and precentrifuged at 500g for 10 min after adjusting the pH to 8.0. The supernatant was recentrifuged at 20,000g for 10 min. This supernatant was adjusted to 10 mM $MgCl_2$, pH 6.8, irradiated for 5 min with red light and then recentrifuged at 50,000g for 30 min. The pellet was resuspended in the extraction medium and the phytochrome content (P_{tot}) was measured in the 50,000g supernatant and 50,000g pellet.

$$\% \text{ Pelletable phytochrome} = \frac{P_{tot} \text{ in 50,000g pellet}}{P_{tot} \text{ in 50,000g supernatant} + P_{tot} \text{ in 50,000g pellet}}$$

P_{tot} was measured at 0 °C with a modified Ratiospect R-2 (ref. 5) using $CaCO_3$ as scattering agent⁶. All irradiations were carried out at 25 °C. Red light was obtained using a standard red light source⁷ (675 W m⁻²). The irradiation with white light was carried out in a phytochamber with xenon arc lamps (7,000 lx).

Fig. 1 Time course of P_{tot} (○) and pelletable phytochrome (●) from squash cotyledons in darkness after induction by white light. The first white light exposure began 4 d after sowing.



In the first set of experiments squash seedlings were grown on Vermiculite for 4 d in the dark at 25 °C. After this germination period seedlings received 12 h white light, were returned to the dark for 12 h and then received a further 12 h white light. P_{tot} and the pelletability were measured in the following dark period every 4 h (Fig. 1). P_{tot} and pelletability were decreased by the white light treatment. In the dark these variables increased, as expected from earlier observation in which plants were irradiated with red or far-red light⁸⁻¹¹. In the experimental conditions phytochrome does not seem to be synthesised by a zero-order reaction^{8-10,12} but P_{tot} and pelletability began to oscillate in anti-phase. After about 36 h in the dark they oscillated in phase. The main oscillation period (observed after using the 'moving means' statistical technique) was roughly 24 h.

To investigate the oscillations of phytochrome pelletability in more detail we used the following light regime. Seedlings were grown in the dark for 4 d at 25 °C and exposed to white light for 12 h. To allow sufficient recovery of phytochrome, the first measurement was made after 2 d darkness. In the following dark period phytochrome pelletability was measured every hour for 2 d. The original data of a representative experiment are shown in Fig. 2a; using the three-point moving mean technique

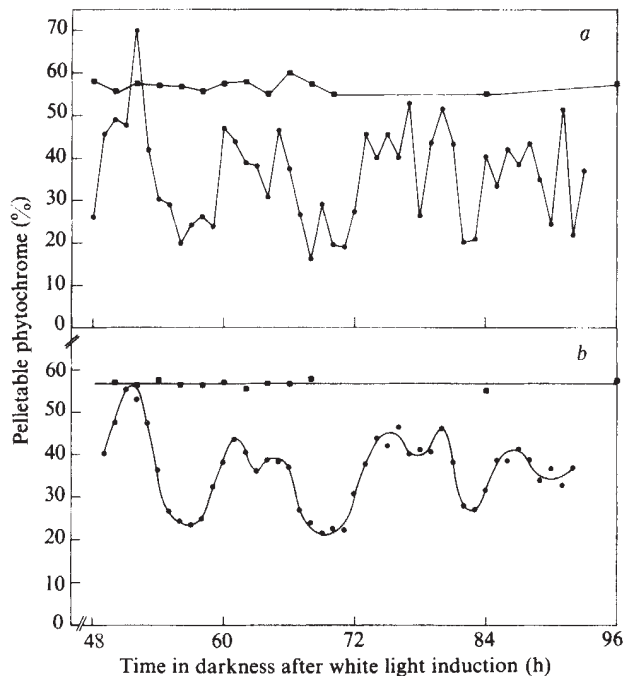


Fig. 2 Time course of pelletable phytochrome from squash cotyledons in darkness after induction by white light (■) or in continuous darkness (●). Seedlings were given only one white light exposure of 12 h.

we obtained the data shown in Fig. 2b. Pelletability oscillated between 20 and 60% and the main period observed was about 12 h. The splitting of the maxima (Fig. 2b) may indicate a second period of about 6 h. The original data indicate the possibility of further oscillations of higher frequencies.

The rhythmic oscillations in P_{tot} and pelletability were almost certainly attributable to the white light induction period. Without this, pelletability remained constant at about 56%, as shown for the dark control in Fig. 2. Similar rhythmic oscillations could be observed by measuring the pH of the crude homogenate before readjusting it to pH 8.0 (Fig. 3). Again a main period of 12 h and a second period of 6 h could be observed. These oscillations of the pH can be regarded as a marker of the endogenous rhythm of the cell. The oscillation of phytochrome pelletability in nearly the same way as pH oscillations indicates that the rhythm of phytochrome pelletability *in vitro* is not a pure artefact of the experimental method¹³.

These experiments demonstrate a rhythmic change in the ability of pelletable structures to bind phytochrome to 'receptor

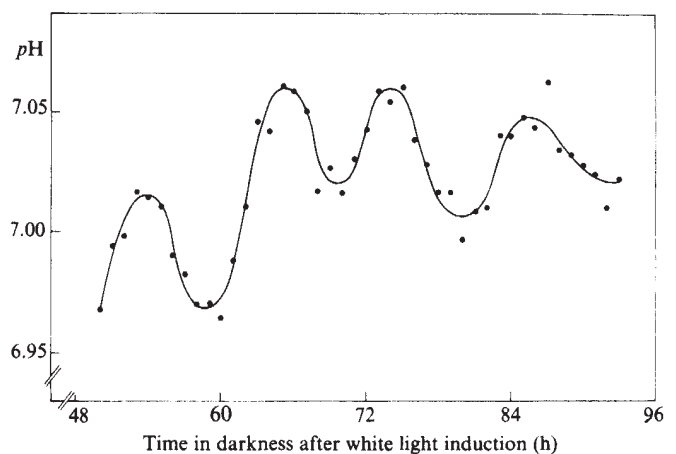


Fig. 3 Time course of changes in pH of crude homogenate from squash seedlings in darkness after induction by white light. Data were calculated using the three-point moving mean technique.

sites'. We cannot decide whether the amount of the pelletable structures or their affinity for phytochrome⁴ changes rhythmically.

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Hormonal control of insect epidermal cell commitment *in vitro*

DEPENDING on the hormonal milieu, the insect epidermal cell can express one of several differentiated states manifest by the type of cuticle it secretes. In the Lepidoptera, as long as the juvenile hormone (JH) titre is high, the epidermis secretes larval cuticle periodically in response to ecdysone. When the JH titre declines at the end of larval life¹, ecdysone causes the secretion of pupal cuticle. In the tobacco hornworm, *Manduca sexta*, two distinct releases of ecdysone are necessary for this metamorphosis^{2,3}. The first triggers the cessation of feeding and causes the epidermal cells to switch from a commitment to make larval cuticle to one for pupal cuticle⁴. After this time JH can no longer prevent pupal differentiation. The second ecdysone release begins 2 d later and causes pupal cuticle synthesis. Consequently, during the time of the first release it is possible to study the events which occur to alter the cellular commitment unhindered by those events involved

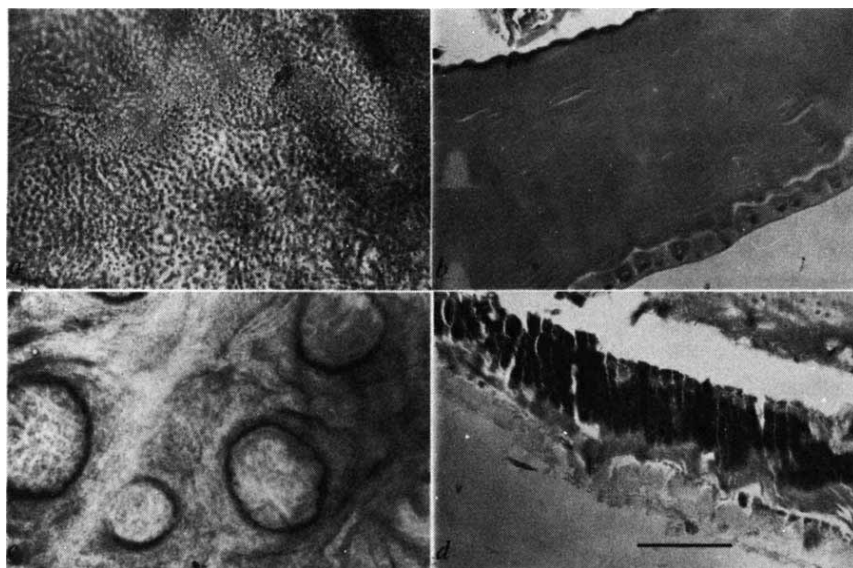


Fig. 1 *a*, Surface view of typical larval cuticle produced by implanted epidermis from a feeding larva (< 7 g), or from an *in vitro* culture without hormones or with both ecdysone and juvenile hormone. *b*, Cross section of a typical larval cyst. *c*, Surface view of tanned pupal cuticle showing typical pockmarks formed by an epidermal cyst after exposure to ecdysone *in vitro* or *in vivo*. *d*, Cross section of a typical pupal cyst. Scale equals 100 μm .

in the expression of this commitment. I report the hormonal manipulation of this cellular commitment in an *in vitro* system.

The dorsal and lateral integument (epidermis and cuticle) from the third to sixth abdominal segments of a 6.2–7.0 g final instar *Manduca* larva was dissected free of fat body and most of the muscle, cut into pieces (approximately $3 \times 5 \text{ mm}$), and explanted to 0.7 ml of Grace's medium (Gibco) in disposable plastic culture wells (Linbro)⁵. β -Ecdysone (Rohto Pharmaceutical) in 10% isopropanol was either premixed with the Grace's medium or added at an appropriate time. C18JH (>95% the natural *trans*, *trans*, *cis* isomer; Eco-Control) was dissolved in Grace's medium by sonication⁶ in a siliconised (Siliclad) vessel. For the JH experiments the tissue was incubated in the JH-containing media for 3–4 h before β -ecdysone was added. The cultures were incubated in a 95% O_2 –5% CO_2 high humidity atmosphere on a slow rotary shaker (12–16 rotations min^{-1}) at room temperature (23–25 $^\circ\text{C}$) for 21–24 h.

The state of commitment of cultured epidermis was then tested by implantation of the pieces into fourth instar larvae before the initiation of the moult to the fifth stage⁷. One or two days after the host had subsequently ecdysed, the implants were recovered. During *in vivo* culture the implanted epidermis grew around its cuticle to form a cyst⁸. During the moult of the host, the epidermis responded to the host's hormonal milieu by forming a new cuticle on the inside of the cyst. The type of cuticle formed reflected the commitment of the epidermal cells at the time of implantation. When epidermis from fourth instar or feeding fifth instar (up to 7 g) larvae was implanted, the cyst formed larval cuticle which was translucent, rubbery and had numerous papillae on its outer surface when viewed at $\times 400$ magnification (Fig. 1*a*). Furthermore, the epidermis was always deeply pigmented (white or blue) as was typical of larval epidermis. Histological sections showed a thick untanned cuticle (Fig. 1*b*) which was identical to cuticle removed from a normal fifth stage larva. In contrast, when epidermis was taken from wandering fifth instar larvae, the cuticle formed was always pupal in spite of the high concentrations of JH in the host⁹. This pupal cuticle was usually hard, shiny, and either tan or white and often had the typical pockmarks of the pupal abdomen (Fig. 1*c*). Figure 1*d* shows this tanning in the cross section of the cyst. Also, epidermis which has formed pupal cuticle no longer contains pigment granules as is typical of pupal epidermis.

Since the cultured epidermis was taken from larvae weighing less than 7 g, 92% ($n=174$) of control pieces

incubated without hormones formed cysts with >90% larval cuticle. When β -ecdysone ($1 \mu\text{g ml}^{-1}$) was added to the epidermis *in vitro*, 92% ($n=81$) of the implants formed pupal cuticle. As the dose of β -ecdysone was decreased, the percentage of cysts forming more than 90% pupal cuticle declined, as shown by the circles in Fig. 2. Many of the cysts from the intermediate doses showed both larval and pupal cuticle; sometimes only tiny spots of pupal cuticle (the size of one to two epidermal cells) were scattered in otherwise normal larval cuticle. Thus, the switchover from larval to pupal commitment seems to be on a cell-by-cell basis as had already been seen in *in vivo* experiments with JH applications^{4,10}. Figure 2 shows that the half-maximal dose of β -ecdysone necessary to commit more than 90% of the cells to pupal differentiation is about $0.1 \mu\text{g ml}^{-1}$ ($2 \times 10^{-7} \text{ M}$). A dose three times smaller ($8 \times 10^{-8} \text{ M}$) is sufficient to alter the commitment in 50% of the cells, as indicated by the triangles in Fig. 2. These amounts of ecdysone are within physiological range; the dose of $1 \mu\text{g ml}^{-1}$ necessary to effect complete switchover in commitment is the maximum amount detected in the animal at the time of the first release of ecdysone³. Also, these data agree well with the concentration of ecdysone necessary to cause evagination in *Drosophila* imaginal disks^{11,12}, development of lepidopteran wing disks^{13,14}, puffing in *Drosophila* chromosomes¹⁵, and moulting of *Manduca* proleg crochets epidermis¹⁶.

The effectiveness of JH in the prevention of this

Fig. 2 Changeover in commitment of larval epidermis as a function of the dose of β -ecdysone for 18–24 h *in vitro*. ●, Percentage of cysts showing > 90% pupal cuticle. ▲, Percentage of cysts showing > 50% pupal cuticle. (15–30 cysts per point, except nine at $0.004 \mu\text{g ml}^{-1}$.)

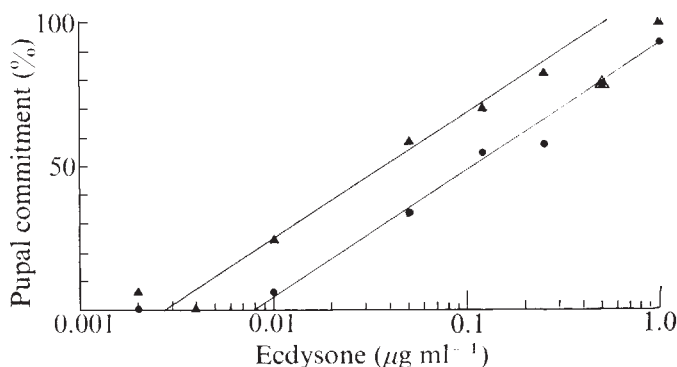
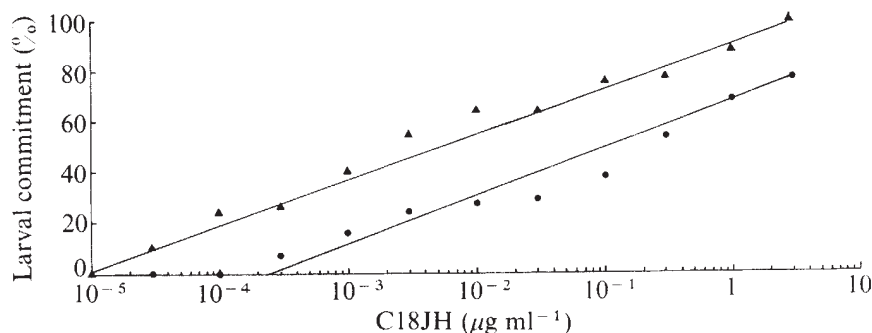


Fig. 3 Inhibition of the changeover in commitment of larval epidermis in response to β -ecdysone ($1 \mu\text{g ml}^{-1}$) as a function of the dose of C18JH *in vitro*. ●, Percentage of cysts showing > 90% larval cuticle. ▲, Percentage of cysts showing > 50% larval cuticle. (15–25 cysts per point, except eight at $0.1 \mu\text{g ml}^{-1}$.)



ecdysone-induced change in commitment *in vitro* is shown in Fig. 3. The amount of C18JH necessary to prevent pupal commitment of the epidermis in response to a concentration of β -ecdysone of ($1 \mu\text{g ml}^{-1}$) was $0.1 \mu\text{g ml}^{-1}$ (3×10^{-7} M). If the cysts showing >50% larval cuticle are considered, then the 50% dose is lowered to 2×10^{-8} M. The concentration of JH in C18JH equivalents in fourth instar *Manduca* haemolymph before the initiation of the larval moult ranges from 5×10^{-7} M to 2×10^{-8} M (ref. 9). Thus, the concentrations of JH necessary *in vitro* are similar to those found in the intact animal. Also, they are two to three orders of magnitude less than those reported to be essential for inhibition of evagination of leg imaginal disks¹⁷ or for inhibition of cuticle deposition in lepidopteran wing disks¹⁸, even in the presence of the JH-binding protein¹⁹. In my cultures little or no JH-binding protein was present because of the virtual absence of fat body which produces it²⁰. Apparently, in *Manduca* this protein is not essential for the cellular action of JH as suggested by Sanburg *et al.*¹⁹. Yet it is possible that the discrepancy found between haemolymph concentration and *in vitro* concentrations could be further reduced by addition of the protein.

This simple *in vitro* system now provides an excellent means for study of the cellular events necessary for the metamorphosis of the insect epidermal cell. When exposed to a physiological dose of ecdysone for 22 h, the cells become committed to pupal differentiation unless a physiological concentration of JH is present. Studies are in progress to elucidate the nature of the cellular events which occur in response to ecdysone. Autoradiographic studies of DNA synthesis in the epidermis during late larval life up to the larval-pupal transformation⁵ showed no detectable DNA synthesis in this dorsal abdominal epidermis until 2 d after the switchover of commitment *in vivo* (on day 4). Instead massive DNA synthesis occurs in response to the second surge of ecdysone^{2,3} the day before cuticle synthesis begins. Thus, the irreversible transformation from larval to pupal determination in the epidermal cells occurs well before DNA synthesis, and presumably reflects some change in regulatory elements in the cell.

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Suckling mouse cataract agent is a helical wall-free prokaryote (spiroplasma) pathogenic for vertebrates

THE suckling mouse cataract agent (SMCA) is a filterable organism originally isolated from a pooled extract of rabbit ticks (*Haemaphysalis leporis-palustris*) collected near Atlanta, Georgia in April 1961 (ref. 1). The agent grows to high titre in the eyes and brains when inoculated intracerebrally into newborn mice in which it induces cataract, uveitis and chronic brain infection^{1–3}. SMCA has also been grown to high titre in embryonated hen's eggs, in which it produces a lethal infection in 4–9 d (ref. 1). The agent has not been grown in tissue culture or in artificial media, but growth in a rabbit lens organ culture has been reported⁴. Stained SMCA-infected tissues failed to show the presence of rickettsiae and, as a result of such negative inferences and other characteristics of the agent^{3–5}, it was considered to be a candidate slow virus⁶. Within the past few years however, ultrastructural and biological studies showed that a wall-free prokaryote was consistently associated with the acute and chronic disease in mice and with lethality in chick embryos^{7,8}. Although the agent resembled mycoplasmas morphologically, it differed from them in its apparent non-cultivability on conventional mycoplasma medium. We now report that the organism associated with the suckling mouse cataract syndrome is a prokaryote similar to the spiroplasmas—a group of mycoplasmas known previously only from plants and insects.

The general designation 'spiroplasma' was first given⁹ to helical, motile, wall-free prokaryotes observed in corn plants infected with the insect-borne corn stunt disease¹⁰, even before cultivation of the agents in artificial media^{11,12}. Similar morphology was also noted for a cultivable mycoplasma recovered from citrus plants infected with "stubborn disease"¹³. Ultrastructural, serological and biological properties of this organism indicated that it deserved recognition as a new genus and species, *Spiroplasma citri*^{13,14}. The absence of cell wall material, and other properties, indicated that this organism belonged to the mycoplasmas (Class Mollicutes). Both of the cultured

spiroplasmas induce disease in insects^{15,16} and plants^{11,12,17}. A third possible spiroplasma has been found in natural populations of four closely related species of *Drosophila*¹⁸. This agent, termed the sex ratio organism (SRO), is inherited maternally and associated with the absence of males in the progeny of infected females¹⁹. SRO spiroplasmas have not been cultured and are not known to be associated with plants or higher animals.

A pool of frozen (-70°C) allantoic fluid from embryonated eggs taken 5–6 d after SMCA infection with about 100 egg lethal doses (ELD_{50}) was passaged in 7-d-old chick embryos. Inoculation (0.1 ml undiluted fluid) was made through the yolk sac into 18 embryos; six uninoculated embryos served as controls. Eggs were incubated at 37°C and candled daily to determine embryo viability.

All embryos receiving SMCA died between 6 and 7 d after inoculation. Uninoculated controls, or embryos inoculated with normal allantoic fluids, were still viable at that time. Allantoic and amniotic fluids were collected, from inoculated and control embryos and examined by darkfield microscopy ($\times 1,250$). Motile, helical microorganisms were observed in allantoic and/or amniotic fluids of all embryos inoculated with SMCA, but were never observed in fluids from control eggs. Numbers of

helical organisms varied from 2 to 5 per microscope field to more than 50–60 per field (Fig. 1a). Continued yolk sac passage of infected allantoic fluids, some containing as few as one helical organism per 10 fields, always yielded fluids containing the motile, helical structures after 3–4 d incubation or when the embryo died. At least 10% of embryonated eggs were used as uninoculated controls in each passage but helical organisms were never seen in fluids of these embryos. The agent was subsequently passaged at least five times each in embryonated eggs from special flocks free of detectable pathogens and mycoplasmas (SPAFAS, Inc., Norwich, Connecticut) or from regular commercial flocks (Truslow Farms, Chestertown, Maryland). No differences in the occurrence or yield of motile, helical organisms were apparent in the two egg sources. Egg passages with selected fresh allantoic fluid, containing numerous helical organisms, reduced the mean day of embryo death from 7.0 to approximately 4.0. Allantoic or amniotic fluids collected 3 d after inoculation, and before most embryos succumbed, were relatively clear and usually contained large number of helices.

We also examined a second, tick-derived, mouse brain-passage isolate (GT-48), which is antigenically indistinguishable from SMCA but causes fatal encephalitis when

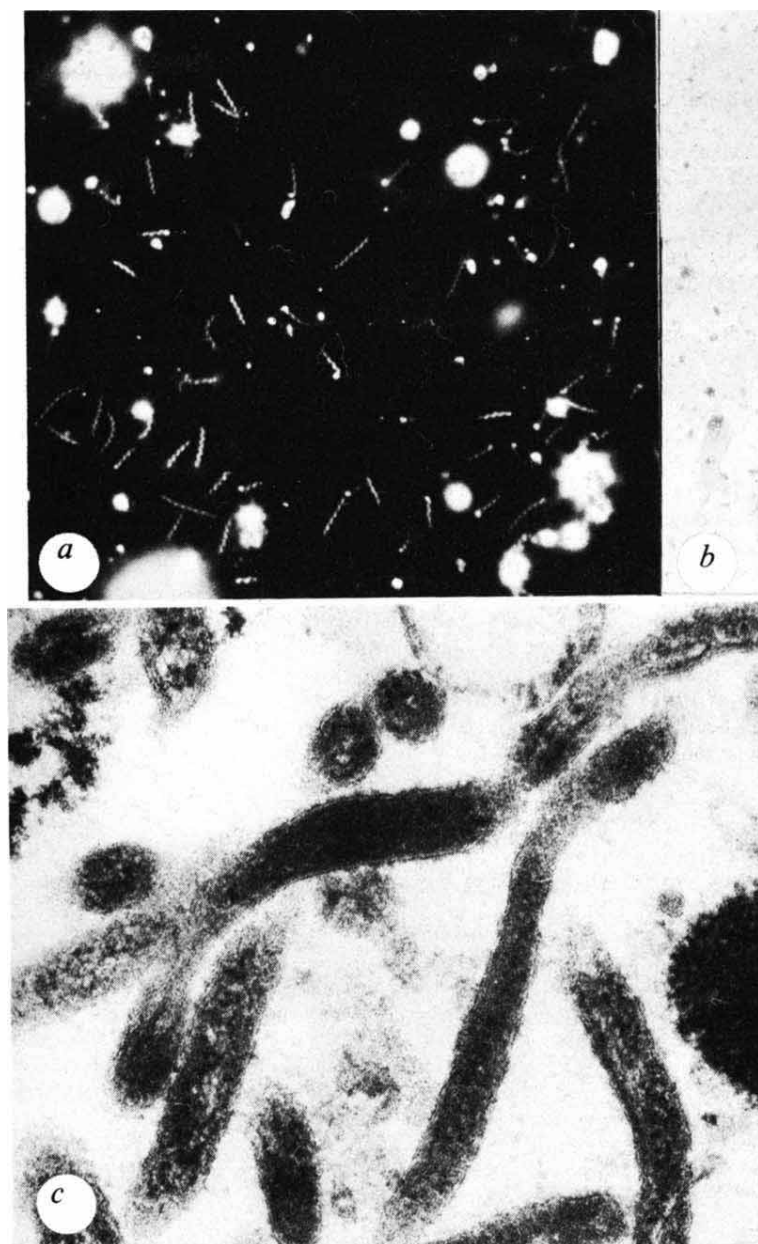


Fig. 1 Appearance of SMCA organisms in chorio-allantoic (CA) fluid of embryonated hen's egg 4 d after inoculation through yolk sac. *a*, Photograph of darkfield preparation of CA fluid showing numerous helical filaments. ($\times 2,800$). *b*, Electron micrograph of negatively stained SMCA. CA fluid was fixed overnight in 1% glutaraldehyde (prepared in 0.15 M NaCl and 0.01 M sodium phosphate buffer, pH 7.2). It was centrifuged at $23,000g$ and the pellet was resuspended in fresh buffered saline. The droplet was picked up on Formvar-coated grid and stained with 1% phosphotungstic acid (pH 7.2). ($\times 26,250$). *c*, Electron micrograph of thin-sectioned pellet of SMCA spiroplasmas. CA fluid was mixed 1:1 with M1 medium¹² and centrifuged at $23,000g$. The pellet was resuspended in fresh M1 medium containing final 1% glutaraldehyde, post-fixed in 1% OsO_4 , dehydrated in graded series of acetone and embedded in Epon. Sections stained with 2% aqueous uranyl acetate and Reynold's lead citrate. ($\times 124,000$.)

inoculated intracerebrally into suckling mice and rats^{1,7}. Pooled mouse brain extracts were passaged three times by yolk sac inoculation. Again, helical organisms were observed in all inoculated eggs and in none of the control embryos.

Negatively stained organisms and thin sections of fixed organisms were examined by electron microscopy (Fig. 1b and c). The organisms varied in length from 3 to 8 μm and from 0.1 to 0.2 μm in diameter. One end was usually more pointed than the other and no axial structure was visible. Electron microscopy of thin sections of pellets containing SMCA showed a typical trilaminar unit membrane with no outer wall or envelope and no axial filaments or flagella.

The possible serological relationship of SMCA-associated spiroplasmas to plant and insect spiroplasmas was assessed by precipitin ring or deformation tests, techniques previously used to study serological properties of spiroplasmas^{18,20}. Preliminary results (Table 1) suggest that the newly discovered spiroplasmas share some antigenic determinants with *S. citri*, corn stunt spiroplasmas, and SRO. These tests also indicate, however, that they are not serologically identical to other described spiroplasmas—findings reminiscent of earlier data on the relationship of *S. citri*, corn stunt organisms and SRO^{18,20}.

The helical organisms from chick embryo-derived SMCA and GT-48 are similar in size to other spiroplasmas (3–12 μm long by 0.1–0.2 μm in diameter)^{9–13,18}. They also possess typical rapid rotary or 'screw' motion and flexional movements, neither of which yields to translational motion. Finally, we have seen no evidence of cell wall, axial filaments or other structures characteristic of spirochetes or other true bacteria. Thus, the term "spiroplasma"

seems appropriate for their nomenclature. To these commonly accepted criteria we add a property shared by SMCA-associated organisms and spiroplasmas—the presence of infecting viruses. Negatively stained organisms showed helices with attached round bodies or sacs containing numerous virions characteristic of those designated SV-C3 (spiroplasma virus-citri 3)²¹.

Our findings provide substantial evidence that fluids from infected eggs and mouse brain extracts of SMCA and GT-48 which induce ocular or central nervous system manifestations in mice and rats contain large numbers of spiroplasmas. Ultimate proof of the association of these spiroplasmas with the pathological conditions noted in these hosts, and assessment of the relationship of these organisms to established species of *Spiroplasma*, will depend on cultivation of the organisms in an artificial medium and subsequent information on their biological, serological and pathogenic properties. Published data on SMCA and GT-48 infections, however, suggest a direct relationship. Physical properties reported for the infectious entity^{1,7,8} correspond closely to expectations for spiroplasmas. Electron microscopic observations on both egg-cultivated SMCA and GT-48 and the involved eye tissues of mice and rats receiving direct challenge with the same egg materials indicated the presence of mycoplasma-like structures⁷. In retrospect, photomicrographs of the agent in both types of tissue showed numerous curved, wall-free organisms similar in structure to spiroplasmas visualised within plant tissue^{10,13}, or to suboptimally-fixed spiroplasmas¹³. We have noted similar structures, again retrospectively, in other micrographs⁵ of SMCA-infected allantoic fluids.

Evidence that SMCA spiroplasmas came from the tick rests primarily on the isolation of two lines of helical organisms from different tick pools, reisolation of the agent from these pools¹, and the absence of similar naturally occurring or latent organisms in pools of other tick species collected at the time¹, or in numerous normal chick embryo fluids examined. The reported isolation²² of a similar agent (277F) from the same tick species adds further support to a tick origin. Although the 277F agent was not pathogenic for suckling mice (Swiss line), and was at first considered to be a spirochaete, the reported properties now leave little doubt that it is similar to SMCA and other spiroplasmas.

Identification of a spiroplasma associated with, and presumably inciting, pathological conditions in vertebrates, extends the host range and pathogenicity of these organisms. The ability of organisms within this group to reproduce at temperatures from 30 to 37 °C and their multiplication and persistence in the eye, brain and other tissues of some vertebrate hosts, suggests that a search for these new microbial forms in diseases of unknown aetiology may be very fruitful.

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Table 1 Serological relationships of SMCA spiroplasmas to other spiroplasmas

Rabbit	Antiserum to	Antigen	Ring precipitation (titre)*	Organism deformation (titre)*
480–481	Pre-immune	SC	<2†	4
	Pre-immune	CSO	<4	<2
	Pre-immune	SMCA	<2	8
	<i>S. citri</i> (SC)	SC	256	5,120
	<i>S. citri</i> (SC)	CSO	64	80–160
	<i>S. citri</i> (SC)	SMCA	32	80
553	Pre-immune	SC	4	4
	Pre-immune	CSO	0	4
	Pre-immune	SMCA	0	16
	Corn stunt organism (CSO)	SC	64	160
	Corn stunt organism (CSO)	CSO	256	2,560
	Corn stunt organism (CSO)	SMCA	64	320
9	Pre-immune	SC	<4	<4
	Pre-immune	CSO	<4	<4
	Pre-immune	SMCA	<4	<2
	SMCA	SC	4	8
	SMCA	CSO	8	4
	SMCA	SMCA	256	640
14	Sex ratio organism (SRO)	SC	4	<2
	Sex ratio organism (SRO)	CSO	4	<2
	Sex ratio organism (SRO)	SMCA	64	10

*Final titre was highest dilution of serum that produced precipitin rings²⁰ or induced deformation of 50% of helices in at least five microscope fields¹⁸. Antigens and antisera prepared from or against cultured spiroplasmas for *S. citri* (SC) and corn stunt organisms (CSO), against SMCA allantoic fluid, and SRO *Drosophila* haemolymph.

†Reciprocal of serum dilution.

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Plaque-forming factor produced by *Mycoplasma pulmonis*

It has been reported that mycoplasma viruses have been isolated from a number of different mycoplasmas—both *Mycoplasma* and *Acholeplasma* species. All the viruses isolated so far, however, only produce plaques on *Acholeplasma laidlawii* lawns and seem unable to infect the mycoplasma from which they were reputedly derived¹. Some strains of *Mycoplasma pulmonis* are pathogenic for mice and others are not, and because pathogenicity may be attributable to the possession of a virus, we have searched for viruses that may infect *M. pulmonis*.

Initially, five strains of *M. pulmonis* were grown and examined for plaque-forming activity by the “washing method” used for the isolation of *Acholeplasma* viruses^{2,3}. All washes were tested on lawns of colonies prepared from each of the mycoplasma strains. In only one case was a

Fig. 1 Growth curve of *M. pulmonis* JB strain and PFF activity
●, *M. pulmonis*; ○, PFF.

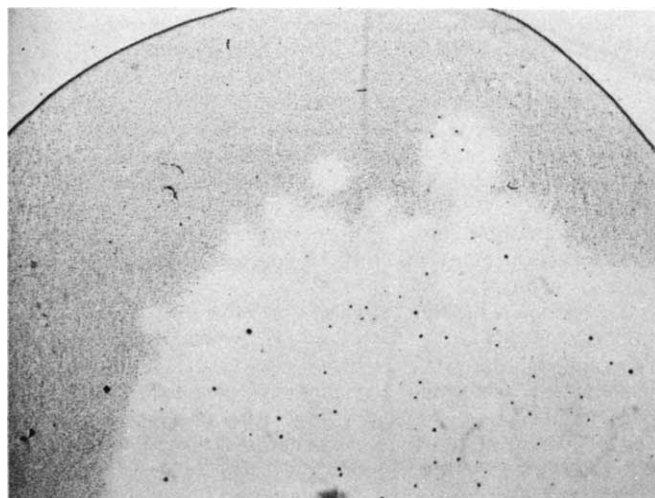
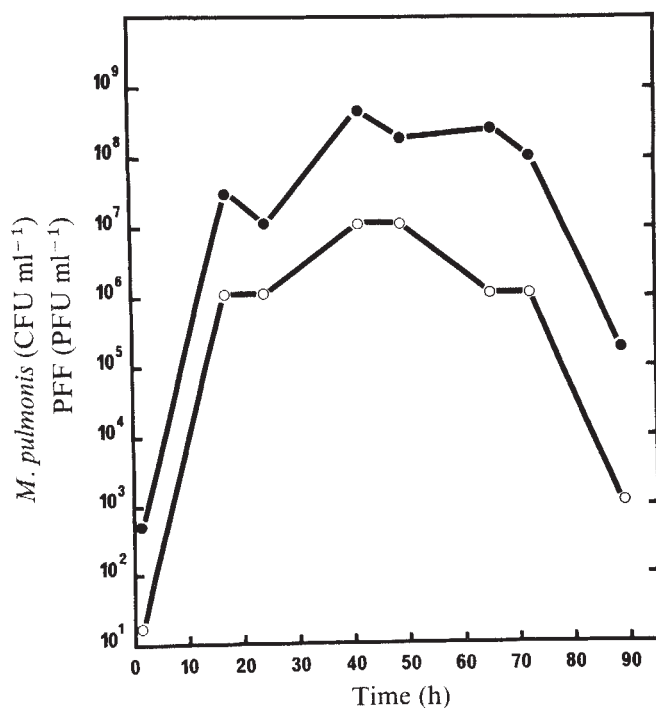


Fig. 2 Plaques produced by *M. pulmonis* JB strain when overlaid with the Peter C strain. Note the mycoplasma colony in the centre of the single plaques.

zone of inhibition or lysis (plaque) seen and this was where the ‘JB’ strain washing had been dropped on the lawn of the ‘Peter C’ strain. The plaque, which extended slightly beyond the area of the drop, was faint but distinct. The titre of the plaque-forming factor (PFF) in the JB wash was found to be 1×10^5 PFU ml⁻¹.

Subsequently, washings from 34 strains of *M. pulmonis* were placed on lawns prepared from 35 strains. Plaques were observed on 96 of the interactions. These plaques were mostly confluent and comprised a central area of the size of the drop, containing mycoplasma colonies (presumably from the washing fluid), and a distinct zone of inhibition around the periphery about 3 mm wide. There were also a few small isolated plaques the largest being only about 1 mm in diameter.

The relationship between mycoplasma and PFF activity was examined by inoculating GS broth⁴ with the JB strain and removing samples at intervals during incubation at 37 °C for mycoplasma counting and assessment of PFF activity. The results (Fig. 1) indicate that the PFF titre corresponds closely with the mycoplasma titre, although about 10- to 100-fold lower. Because of the difficulty of counting the small plaques, we devised a more accurate method of examining the relationship between mycoplasmas and PFF activity. Cultures obtained from all of 90 single colonies grown from filtered (450-nm) cultures showed plaque-forming activity, suggesting that as each colony was probably derived from a single mycoplasma cell, each cell therefore possesses plaque-forming activity.

Larger, more distinct plaques were formed when the JB strain was preincubated before being overlaid with the Peter C strain. The inhibition area round the JB colonies increased in size with the length of preincubation until it reached a diameter of 6 mm after 7 d. Where single plaques were visible, one mycoplasma colony, slightly larger than those constituting the lawn, was observed in the centre of each plaque (Fig. 2).

We then investigated the possibility that the area of agar comprising the plaque contained viable mycoplasma organisms. Colonies of JB strain preincubated for 3 d were overlaid with a culture of Peter C strain. After further incubation, plaques 2–3 mm wide developed round the JB colonies. A duplicate which had not been overlaid with Peter C was used to pick samples of agar 0.5–1 mm from the edge of the JB colonies; these samples and the colonies themselves were tested for viable mycoplasmas. Myco-

plasmas were not isolated from the plaques but they were isolated from the colonies.

The plaques may have resulted from inhibition by acid, produced by the JB colonies, so we compared plaque production on normal GS agar and GS agar maintained at pH 8.5 with HEPES buffer. Plaques were produced equally well on both types of agar. It also seemed unlikely that peroxide production by the JB colonies was responsible for plaque formation as plaques developed in an atmosphere of 95% N₂-5% CO₂. The failure of catalase (Boehringer), incorporated in agar medium at a final concentration of 10,000 U ml⁻¹, to inhibit plaque development confirmed this.

We tried to transmit the PFF, but washings of plaques produced by JB on Peter C lawns produced plaques that were fainter than the original ones and single plaques picked with a straight wire generally failed to produce further plaques.

The size of the PFF was determined by serial filtration of a JB culture under positive pressure of 5-10 pound inch⁻², through Millipore membranes of decreasing pore size. The mycoplasma titre of the culture before filtration was 8×10^8 colony-forming units (CFU) per ml, and 5×10^7 CFU ml⁻¹ and 1×10^5 CFU ml⁻¹ after filtration through 650-nm and 450-nm membranes, respectively. The titre of the PFF was initially about 2.5×10^7 PFU ml⁻¹ and 5×10^6 PFU ml⁻¹ and 5×10^3 PFU ml⁻¹ after filtration through the respective membranes. Neither mycoplasmas nor plaque-forming activity were detected in the 220-, 100- or 50-nm filtrates.

The filtrates were also examined for their ability to inhibit growth of the Peter C strain in a metabolism inhibition test. No inhibition of growth was detected with the 50-nm, 100-nm and 220-nm mycoplasma-free filtrates. The 450-nm and 650-nm filtrates contained too many mycoplasmas for the test to be useful.

A culture of the JB strain, diluted twofold in phosphate-buffered saline (PBS), was held at 56 °C and viable organisms and PFF activity were determined at intervals. As shown in Fig. 3a, the rate of PFF inactivation was about the same as the rate of organism death. A sample (5 ml) of the same JB culture used for heat inactivation was centrifuged and the deposit resuspended in 0.25 ml of PBS. The suspension was diluted 100-fold in PBS, treated with ultraviolet light, and samples taken at intervals for estimation of mycoplasma viability and PFF activity. The results (Fig. 3b) indicate that when the mycoplasmas died, PFF activity was lost. Treatment with Nonidet P-40 (0.4%) for 15 min at 37 °C inactivated the PFF activity. The titre before treatment was 1×10^6 PFU ml⁻¹ and $< 2.5 \times 10^3$ PFU ml⁻¹ after treatment. Samples of a culture of strain JB taken at intervals were incubated at 37 °C for 7 h with Mitomycin C (Sigma) or Kanamycin (Kannasyn, Winthrop), at final concentrations of 1 µg ml⁻¹ and 5 µg ml⁻¹, respectively, after which they were examined for mycoplasma viability and PFF activity. Mitomycin C and Kanamycin abolished both mycoplasma viability and PFF activity. To examine the specificity of the plaque-forming activity of the JB strain, tenfold dilutions of a JB culture were placed on GS agar and preincubated at 37 °C for 4 d. Cultures of *A. laidlawii* (strains M1305/68 and BN1), *A. granularum* (Switzer), *A. modicum* (M45-67-1), *M. bovirhinis* (56R and 190), *M. hominis* (3477/44 and 1100/73), *M. gallisepticum* (S6 and A5969) and *M. arthritis* (PG6 and PG27) were poured over the surface of the different agar plates which were reincubated. Confluent lawns developed with all the mycoplasmas, but plaques only developed around the JB colonies overlaid with the S6 strain of *M. gallisepticum*. Centrifuged deposits of a 48-h broth culture of JB strain were examined under the electron microscope by negative staining, or by touch preparations prepared by methods described pre-

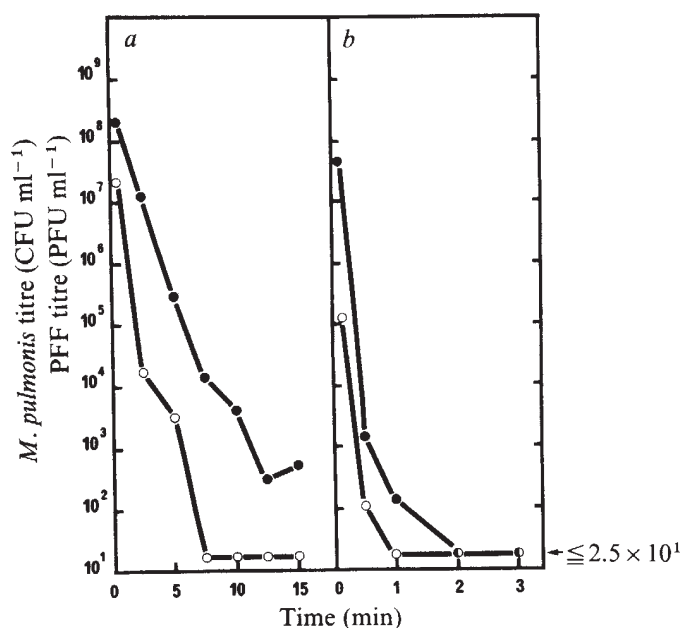


Fig. 3 Inactivation of *M. pulmonis* JB strain and the PFF, by a, heat (56 °C) and b, ultraviolet light treatment. ●, *M. pulmonis*, ○, PFF.

viously⁵. Spherical virus-like particles, some about 15 nm in diameter and others about 50 nm in diameter, were frequently observed among the mycoplasma cells. Sections of agar within plaques were also examined but no virus-like particles were seen, so no proof was obtained that the virus-like particles among the mycoplasma cells were associated with plaque-forming activity.

The formation of a plaque on a lawn of the Peter C strain is dependent on the presence of a colony of the JB strain in its centre. In addition, the PFF is inactivated by treatments which also inactivate the JB mycoplasma. The plaque-forming activity also seems to be directly related to the titre of viable mycoplasma organisms as shown by growth and inactivation curves. These observations indicate that the PFF does not develop in the absence of growing JB mycoplasmas. It is intimately associated with the viable JB mycoplasma cell and seems to be inseparable from it. The area of inhibition around a JB colony extends beyond its periphery, however, suggesting that inhibition is due to some diffusible material elaborated by the colony, even though the filtration studies indicate that the apparent size of the PFF is > 220 nm. There is no evidence that such diffusible material is either acid or hydrogen peroxide. The PFF does not have the same characteristics as the mycoplasma inhibitors described by Altucci *et al.*⁶ and Furness⁷. Although the PFF seems to have selective activity against certain *M. pulmonis* strains (and *M. gallisepticum*, S6) the inability to propagate plaques serially and to find virus particles within them suggests that plaque formation is not likely to be due to virus production by *M. pulmonis*. The possibility that the PFF is a readily inactivated bacteriocin requires further investigation.

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Enzymatic basis for DDE-induced eggshell thinning in a sensitive bird

ALTHOUGH the thinning of eggshells as a result of ingestion of DDE [2,2-bis-(*p*-chlorophenyl)-1,1-dichloroethylene] is well documented in many sensitive avian species, the underlying mechanism remains unclear (see ref. 1 for review). Risebrough *et al.*² have proposed that thinning results from inhibitory action close to the site of shell formation since other physiological processes are apparently not affected in brown pelicans laying eggs with up to 95% thinned shells. Our report of no reduction in blood calcium in white Pekin ducks and ring doves exhibiting DDE-induced eggshell thinning³ supports the proposal that thinning results from a disruption of shell gland (uterus) function rather than a reduced calcium supply to the gland.

The primary function of the avian shell gland is to secrete calcium and carbonate during eggshell formation. Since large quantities of these substances are not stored in the gland, they must be obtained from the blood (calcium) or from metabolic CO₂ (carbonate) and transported to the calcifying egg⁴. As with other tissues which actively transport calcium, for example the intestine⁵, avian shell gland mucosa possesses both calcium-dependent adenosine triphosphatase (Ca-ATPase)^{6–8} and calcium binding protein (CaBP)⁹. Mediators of carbonate (or bicarbonate) transport in this tissue include carbonic anhydrase (CA)⁴ and, possibly, anion (HCO₃)⁻-ATPase¹⁰; other transported-related enzymes include Na,K-ATPase⁶ and Mg-ATPase⁶, a part of which we have found to be oligomycin sensitive. The inhibition of one or more of these transport enzymes by DDE could account for the shell thinning observed in sensitive species. Cooke¹ has suggested that there may be several shell thinning mechanisms and that the relative importance of each is species dependent. We have now found that DDE-induced eggshell thinning in ducks is accompanied by decreases in the activities of two shell gland enzymes, Ca-ATPase and CA, the first apparently more important.

Laying white Pekin ducks (which are relatively sensitive to DDE¹¹) approximately 9–12 months old were divided into experimental and control flocks and maintained as in ref. 11. White leghorn hens (which are relatively insensitive to DDE and provide a natural control²) approximately 12 months old were assigned to control or experimental treatments and maintained in individual cages. Experimental feed was prepared by adding DDE (40 p.p.m.) to mash before making it into pellets. Eggs were collected daily and shell thickness was measured as in ref. 12. To determine shell gland enzyme activities and DDE residues, birds with a calcifying egg in the shell gland were decapitated and the gland was excised. After rinsing in ice-cold, 0.25 M sucrose the mucosa was scraped and the scrapings were homogenised (10–20% weight per volume) in 0.25 M sucrose. Aliquots were frozen rapidly in a dry ice-acetone bath, freeze dried at –20 °C for 24–48 h and stored at –20 °C until use. The ATPase activity in freeze-dried homogenates was determined using a modification of Bonting's method¹³ (Fig. 1 legend gives assay conditions); that of CA in freeze-dried homogenates using the method of Maren¹⁴; that of CaBP in fresh homogenates using the Chelex procedure of Wasserman and Taylor¹⁵; DDE residues in dried tissue samples were determined using the method of Cade *et al.*¹⁶. Protein was determined using the

procedure of Lowry *et al.*¹⁷. As Bonting¹³ has noted, and we have confirmed for shell gland mucosa, freeze drying releases maximal ATPase activity from homogenates, and tissue treated in this manner can be stored at –20 °C for at least three months with no loss of enzyme activity. Pritchard *et al.*⁸ have obtained a low Ca-ATPase activity in fresh shell gland homogenates from duck, and Pike and Alvarado⁸ have observed a loss of Ca-ATPase activity after storage of fresh shell gland microsomes from quail at 0 or –20 °C for 24 h.

Freeze-dried whole homogenates of duck shell gland mucosa had the following ion-activated ATPases: Ca-ATPase, Mg-ATPase, Na,K-ATPase and anion (HCO₃)⁻-ATPase. As Fig. 1 shows, the addition of DDE to the ATPase assay medium substantially inhibited all ATPase activities. Their *in vitro* sensitivity to low DDE concentrations varied markedly, however, with only Ca-ATPase and Na,K-ATPase inhibited below 3 p.p.m. The fractionation of homogenates before freezing (method of Homan and Schraer¹⁸) revealed that the most sensitive Ca-ATPase activity was associated with mitochondria (Fig. 2). These *in vitro* dose-response data are perhaps significant in view of the low gland DDE residues (0.75–1.3 p.p.m.) in ducks

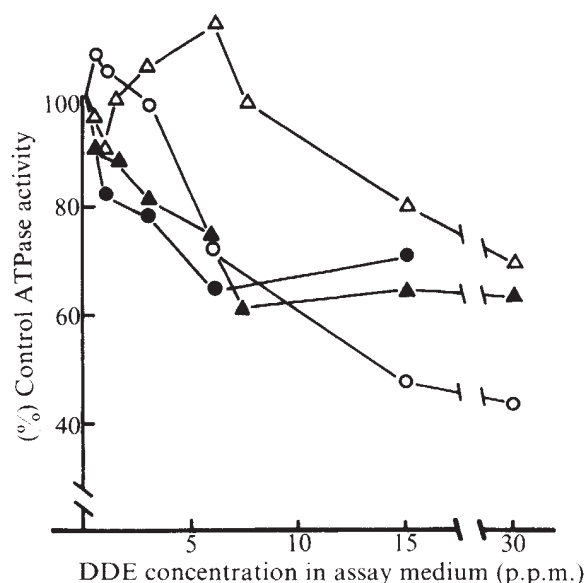


Fig. 1 Inhibition of ion-activated ATPases in duck shell gland mucosa by DDE added *in vitro* to the assay medium. Each point represents the mean of 3–12 ATPase assays on pooled whole homogenates from 2–4 control ducks. Freeze-dried homogenates were reconstituted in 0.25 M sucrose and 0.2 ml aliquots were added to test tubes containing 0.6 ml buffer and salt solutions. ATPase activity expressed as the rate of inorganic phosphate liberation at 37 °C in *V*_{max} conditions after correction for non-enzymatic ATP hydrolysis. Five incubation media were used: A, 5 × 10⁻³ M CaCl₂, 3 × 10⁻³ M ATP, 92 × 10⁻³ M Tris (pH 7.7); B, 100 × 10⁻³ M NaCl, 10 × 10⁻³ M KCl, 3 × 10⁻³ M MgCl₂, 3 × 10⁻³ M ATP, 92 × 10⁻³ M Tris (pH 7.7); C, medium B, except with 0.1 × 10⁻³ M ouabain; D, 3 × 10⁻³ M MgCl₂, 2 × 10⁻³ M ouabain, 69 × 10⁻³ M Tris (pH 7.6); E, medium D, except with 30 × 10⁻³ M Na₂SO₃. Ca-ATPase activity was determined in medium A; Mg-ATPase in medium B; Na,K-ATPase as the difference in activity measured in media B and C; anion-ATPase as the difference in activity measured in media D and E. For the anion-ATPase, sulphite was used as it yielded a proportionately greater activity than bicarbonate. The reaction was started by the addition of 0.2 ml of a solution of 15 × 10⁻³ M ATP and was terminated 10 min later by the addition of 4 ml of ice-cold colour reagent, a strongly acidic solution of 1% ammonium molybdate in 1.15 N H₂SO₄ in which 40 mg ml⁻¹ ferrous sulphate had been dissolved before use¹³. After centrifugation, absorbance was measured at 700 nm. DDE was added to the assay medium in *n,n*-dimethylformamide. The final solvent concentration was 0.5–1%; this concentration did not affect enzyme activity. Control rates of ATP hydrolysis were measured in the presence of solvent alone. △, Mg-ATPase; ○, anion-ATPase; ▲, Ca-ATPase; ●, Na,K-ATPase.

Table 1 Effect of DDE feeding on duck shell gland ion-activated ATPases and CaBP

	Control	40 p.p.m. DDE	
	4 d	4 d	1-3 months
Shell thickness (mm)	0.483±0.008 (37)*	0.422±0.014 (5)†	0.395±0.008 (17)†
Ca-ATPase	16.5±0.9 (12)	12.6±1.1 (5)†	11.4±0.9 (7)†
Mg-ATPase	25.5±1.7 (12)	21.4±1.5 (5)	21.7±2.2 (7)
Na,K-ATPase	3.9±0.5 (12)	2.3±0.4 (5)‡	4.4±0.7 (7)
HCO ₃ -ATPase	4.6±0.4 (11)		4.8±0.6 (4)
CaBP	5.0±1.1 (5)		4.1±0.7 (4)
Carbonic anhydrase	19.5±1.2 (6)		15.8±0.7 (6)‡

* Values given as mean ± s.e. (*n*), where *n* is the number of eggs or ducks (enzyme data). Data expressed as mm for shell thickness, μmol Pi released per mg protein per h for ATPases, moles Ca bound per mg protein for CaBP and enzyme units per mg protein for CA. ATPase assay conditions as in Fig. 1, except that HCO₃-ATPase was determined in media D and E with 25 × 10⁻³M NaHCO₃ replacing sulphite in medium E.

† Significantly different from controls, *P* < 0.01.

‡ Significantly different from controls, *P* < 0.05.

laying thin-shelled eggs (Fig. 3). In contrast, Ca binding to gland CaBP was only inhibited by *in vitro* DDE concentrations above 10 p.p.m.: for example, 30% at 27 p.p.m. Finally, Maren *et al.*¹⁹ have reported that there is no inhibition of duck shell gland CA with *in vitro* DDE concentrations as high as 150 p.p.m.

To determine whether duck shell gland enzymes were affected *in vivo*, we fed 40 p.p.m. DDE to laying birds. In agreement with earlier work¹¹, this dietary level caused 13±4% (mean±s.e.) shell thinning after 4 d and 18±3% after 1-3 months (Table 1). Thinning was maximal after about 1 week and then remained constant. The activity of Ca-ATPase in whole gland homogenates from these ducks was reduced 24±10% after 4 d and 31±10% after 1-3 months (Table 1) with no tendency to change during the 1-3 months. The only other significant effects (Table 1) were a transient reduction in Na,K-ATPase activity (41±22% after 4 d), which seems to be unrelated to shell thinning because of the return to normal after 1-3 months, and a limited reduction in CA activity (19±8% after 1-3 months), which exceeds that reported previously⁶ (10±4% after 2-3 weeks) for ducks fed 40 p.p.m. DDE.

In contrast to the duck, the activity of Ca-ATPase in chicken shell gland was not inhibited by *in vitro* DDE concentrations up to 15 p.p.m. (Fig. 2). Moreover, 2 weeks of feeding 40 p.p.m. DDE did not affect eggshell thickness, any ATPase, or CA (Table 2), even though gland residues were comparable to those found in ducks laying maximally thinned eggshells (Fig. 3). Finally, to determine whether the Ca-ATPase in duck intestine was also unaffected *in vivo*, we fed 40 p.p.m. DDE to male ducklings for 4 weeks. As Table 3 shows, neither Ca-ATPase nor Na,K-ATPase activities were altered, although DDE residues averaged 1.4±0.3 p.p.m. (wet weight whole intestine).

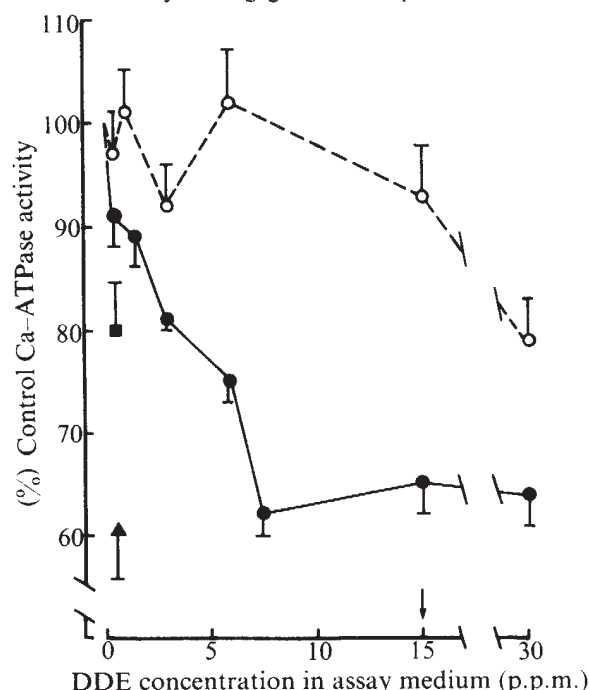
Table 2 Effect of DDE feeding on chicken eggshell thickness and shell gland ATPase activity*

	Control	40 p.p.m. DDE
Shell thickness (mm)	0.398±0.007 (12)	0.405±0.006 (12)
Ca-ATPase	12.8±0.8 (11)	11.5±0.6 (12)
Mg-ATPase	21.7±1.9 (6)	23.6±1.8 (6)
Na,K-ATPase	3.1±0.6 (6)	2.4±0.8 (6)
HCO ₃ -ATPase	3.8±0.4 (6)	4.8±1.4 (6)
Carbonic anhydrase	25.4±2.1 (6)	24.7±3.7 (6)

* Experimental birds fed 40 p.p.m. DDE for 2 weeks. Values given as mean ± s.e. (*n*), where *n* is the number of eggs or chickens. Units and assay conditions as in Table 1. Shell data are for eggs collected on the last day of the experiment; additional data (not shown) indicated no change in control or experimental shell thickness during the experiment.

Whether CA, a soluble enzyme known to be involved in eggshell formation, can be inhibited directly by water-insoluble organochlorines is a matter of controversy¹. Certainly, duck shell gland CA is insensitive to DDE *in vitro*¹⁹ and the 10-20% decrease associated with maximal shell thinning *in vivo* (ref. 6 and Table 1) may reflect DDE action at cellular sites controlling enzyme synthesis or degradation. Moreover, since CA in many tissues seems to be present in great excess²⁰, 10-20% reduction in the activity of duck shell gland suggests a minor role for CA in shell thinning caused by DDE. This mechanism may be

Fig. 2 Inhibition of Ca-ATPase activity in whole homogenates of duck (solid line) and chicken (dashed line) shell gland mucosa by DDE added *in vitro* to the assay medium. Also included are data for crude mitochondrial (▲) and microsomal (■) fractions of duck homogenate. Each point represents the mean of 4-12 assays on pooled homogenates or fractions from 2-4 control ducks or 4 control chickens; variability, when large enough, is given by s.e. bars. Assay conditions as in Fig. 1. Mitochondrial Ca-ATPase activity was reduced to 30% of control by 15 p.p.m. DDE; control activity in both fractions was 3-4 times higher than in whole duck homogenate. Fractions were prepared by differential centrifugation¹⁸ before freezing, and characterised by the presence of generally accepted marker enzymes: oligomycin-sensitive Mg-ATPase for mitochondria and Na,K-ATPase for microsomes. ATPase activity was negligible in the supernatant fraction.



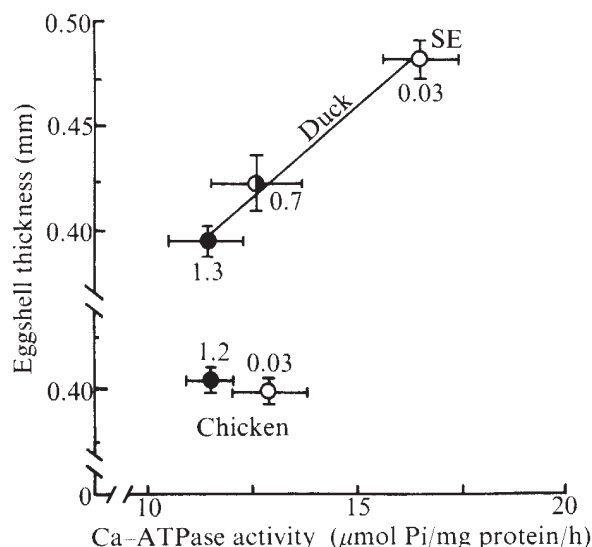


Fig. 3 Difference in *in vivo* sensitivity to both Ca-ATPase inhibition and eggshell thinning following DDE feeding (40 p.p.m.) in ducks (5–12 birds) and chickens (11–12 birds). Residues are means of values obtained from 4–5 birds. Control duck residues taken from ref. 11. ○, Control; ●, 4 d; ●, ≥ 2 weeks. Figures show DDE residues (p.p.m.) in shell gland mucosa.

more important in other sensitive species, such as the ring dove, in which Peakall²¹ has reported a 60% reduction in gland CA activity during maximal DDE-induced shell thinning.

In contrast, all the available evidence points to a major role for Ca-ATPase inhibition by DDE in the shell gland of the duck. Organochlorines often inhibit membrane-bound enzymes such as the ATPases²², and the correspondence between *in vitro* and *in vivo* sensitivities to DDE in ducks and chickens (Figs 2, 3) suggests direct Ca-ATPase inhibition with the duck shell gland enzyme being particularly sensitive for unknown reasons. Moreover, unlike CA, transport enzymes such as Na, K-ATPase and Ca-ATPase do not seem to be present in excess, and partial inhibition is generally accompanied by a parallel reduction in ion transport^{13,23,24}. Thus, the close correlation between

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Exposure by phospholipase A of receptors for sheep erythrocytes on human B cells

HUMAN T lymphocytes are known to form rosettes (E rosettes) with certain specific xenogeneic erythrocytes, including sheep erythrocytes (SRBCs). This rosette formation has been widely used as a specific marker for T cells and valuable information has been obtained^{1–3}. There is, however, increasing evidence that multiple factors influence the rosette formation; some of these factors can be related to an external effect^{4,5} or to the active cell metabolism^{6,7}, whereas others are more intimately related to the processes operating during cell maturation⁸. Further, the presence of lymphoid cells carrying E rosette receptors, as well as markers for B cells, has been demonstrated⁹. It has also been demonstrated that neuraminidase treatment induced E rosette formation in a population of lymphocytes bearing B-cell characteristics¹⁰. Thus, it has not yet been established whether the 'presence of receptor' for E rosette is specific for T cells or not. For this reason, an elucidation of the factors that influence the receptor activity is important if the exact nature of the receptor and the population of cells with it is to be clarified. In the course of investigations on such factors, we have found that treatment of the cells with phospholipase A (PLA) from cobra venom induced the ability to form E rosette in human lymphoblastoid cells that

Table 3 Effect of DDE feeding on duckling intestinal ATPase activity*

	Control	50 p.p.m. DDE
Ca-ATPase	8.35 ± 0.77 (7)	9.07 ± 0.92 (5)
Na,K-ATPase	3.41 ± 0.23 (9)	3.20 ± 0.92 (9)
Mg-ATPase	11.8 ± 1.2 (9)	15.7 ± 0.7 (9)†

* Birds fed 40 p.p.m. DDE for 4 weeks. Values given as mean ± s.e. (n), where n is the number of ducklings. Units and assay conditions as in Table 1.

† Significantly different from controls, *P* < 0.05.

Ca-ATPase activity and eggshell thickness in ducks (Fig. 3) is consistent with a cause and effect relationship. Finally, although few details of calcium transport in the avian shell gland are known, the mitochondrial accumulation of calcium seems to be involved²⁵, and the *in vitro* sensitivity of the Ca-ATPase associated with this organelle (Fig. 2) suggests that DDE, in fact, inhibits a calcium pump in duck shell gland mitochondria.

We thank David G. Shoemaker, Martha Winsor and Allyn Seymour Jr for technical assistance. This study was supported by the US Public Health Service and the National

are known to be B-cell line. The study also indicated that a population of presumed B cells from the peripheral blood also formed rosettes after such treatment.

PLA was purified from lyophilised powder of cobra (*Naja naja*) venom (Sigma) according to the method described by Lankish *et al.*¹¹. The purified preparation used had an activity unit of 20.8 μmol of released fatty acid per min per mg as assayed using phosphatidylethanolamine (PE) from *Escherichia coli*¹². The preparations were free from detectable toxic basic proteins and anticomplementary, neuraminidase and protease activities. Human and marmoset lymphoblastoid cell lines were cultivated in RPMI-1640 medium containing 10% foetal calf serum (FCS). Lymphocytes were isolated by Ficoll-Isopaque centrifugation of defibrinated peripheral blood.

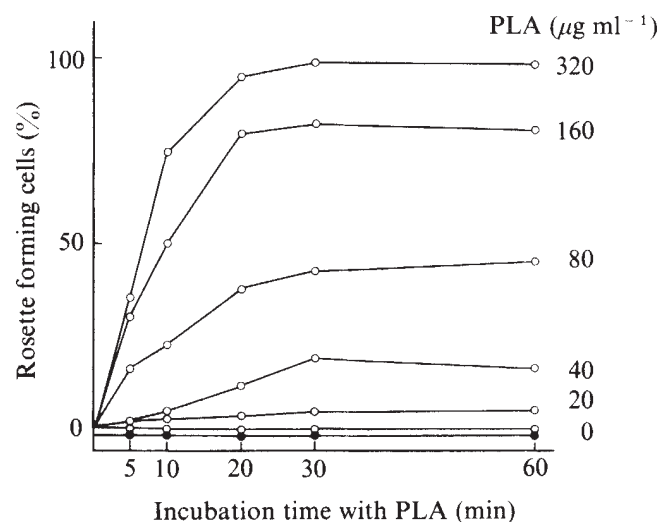


Fig. 1 Rosette formation in Daudi cells treated with PLA. Approximately 1×10^6 Daudi cells were incubated at 37 °C in the Tris-HCl buffer, pH 7.2 containing PLA at the indicated concentrations for varying times, washed twice with GVB and then tested for the ability to form rosettes by SRBCs. 0.1 ml of $1 \times 10^6 \text{ ml}^{-1}$ Daudi cell suspensions were mixed with 0.1 ml of 1.0% SRBCs in the GVB, incubated at room temperature for 5 min and spun at 1,000 r.p.m. for 5 min. After 3 h incubation at 1 °C, the pellet was gently resuspended and the percentages of rosette forming cells (RFCs) were measured (○). Although only the cells with three or more SRBCs were evaluated as RFCs, almost all the RFCs usually had many red cells. All rosette assays were done in duplicate. As a control for 37 °C, Daudi cells treated with 320 $\mu\text{g ml}^{-1}$ of PLA at 4 °C for varying times were tested for the ability to bind SRBCs (●). In this case, even overnight incubation with PLA did not induce rosette formation in the cells.

Daudi cells, as a representative of the human B lymphoblastoid cell lines, were incubated at 37 °C for varying times in the presence of PLA at different concentrations, washed with gelatine Veronal buffer (GVB) and tested for rosette formation by SRBCs. Daudi cells after being treated with PLA, apparently formed rosettes by SRBCs (Fig. 1). The percentages of cells capable of binding SRBCs rapidly increased within 20 min of incubation in the PLA at any concentrations greater than 40 $\mu\text{g ml}^{-1}$ and reached a plateau at 30 min. At that time almost all the cells treated with 320 $\mu\text{g ml}^{-1}$ of PLA could form rosettes. Overnight incubation of Daudi cells in the PLA at 1 °C, however, did not induce any rosette formation. When the SRBCs were incubated at 37 °C for 1 h in the 320 $\mu\text{g ml}^{-1}$ PLA and then tested for their ability to adsorb on the non-treated Daudi cells, no rosette formation was observed. Further, when the mixture of Daudi cells and SRBCs was incubated overnight in the presence of the enzyme at 1 °C and tested for the occurrence of rosettes in the mixtures, no

Table 1 Rosette formation with SRBC in various tissue culture cells treated with PLA

Cells	Species	Types	% of rosettes§
Daudi*	Human	Lymphoid	95 $\pm$ 4
HR 1*	Human	Lymphoid	97 $\pm$ 2
Raji*	Human	Lymphoid	99 $\pm$ 1
HL 2*	Human	Lymphoid	98 $\pm$ 2
MOLT 4†	Human	Lymphoid	96 $\pm$ 1
MOLT 4F†	Human	Lymphoid	98 $\pm$ 2
HeLa‡	Human	Epithelial	0
HEL‡	Human	Fibroblast	0
B95-8*	Marmoset	Lymphoid	0
LLCMK-2‡	Monkey	Kidney	0
RK-13‡	Rabbit	Kidney	0

* Before PLA treatment, no definite RFC were observed.

† These cell lines have been shown to possess the ability to form rosettes¹³. During the passage, however, the cell lines showed decreased ability to form rosettes. At the present time we cannot detect > 0.1% of RFCs in the culture without pretreatment by PLA, although other characteristics of the cells such as lacking of detectable EBV genome remain unchanged.

‡ These monolayer cells were cultivated in Eagle's MEM containing 10% calf serum. Monolayers were split with trypsin, washed twice with GVB and then treated with PLA. No definite RFCs were observed before PLA treatment.

§ Cells were treated with 320 $\mu\text{g ml}^{-1}$ PLA at 37 °C for 30 min and then tested for rosette formation. Values represent means $\pm$ s.d. in 2-3 different experiments.

rosettes were found. Heating the enzyme at 100 °C for 15 min at pH 8.5 mostly abolished the rosette-inducing activity of the enzyme, whereas heating at 100 °C for 15 min at pH 6.0 did not change the activity. Thus, it can be concluded that the rosette induction may be produced by direct enzymatic action on the Daudi cells, but may not be produced by the effect on erythrocyte membrane or by the agglutinin-like effect bridging across both the cells because of its nonspecific binding to the cell membranes.

The induction of rosette formation by the PLA was observed only in human lymphoblastoid cell lines. Table 1 shows that all of six human lymphoblastoid cell lines including two MOLT cell lines formed rosettes. In contrast, no rosettes could be formed in the other cell lines regardless of the cell species and types. This suggests that the induction of rosette formation with SRBCs by the PLA may be restricted to the lymphoid cells of human species.

The species specificities of indicator erythrocytes capable of adsorbing on the PLA-treated Daudi cells were tested. The lymphocytes from peripheral blood were also tested for rosettes formation by the same series of erythrocytes (Table 2). Sheep, pig and mouse erythrocytes formed rosettes in 53-70% of the lymphocytes which could be included in the T-cell population. This specificity of the

Table 2 Comparison with the specificities of erythrocytes capable of adsorbing on the PLA-treated Daudi cells and on the non-treated peripheral blood lymphocytes

Erythrocytes	Daudi*	% Rosettes Lymphocytes†
Sheep	100	70
Pig	100	53
Mouse(dd)	100	68
Human (O)	0	0
Rabbit (albino)	0	0

* Daudi cells were treated with 640 $\mu\text{g ml}^{-1}$ PLA at 37 °C for 30 min. The washed cells were then tested for the rosette formation by the indicated erythrocytes as described in the footnote of Fig. 1.

† For the rosette assay of lymphocytes, 1×10^6 - $2 \times 10^6 \text{ ml}^{-1}$ lymphocyte and 1.0% erythrocyte suspensions were respectively made in the foetal calf serum previously adsorbed with the indicated erythrocytes. 0.1 ml of each suspension was mixed, incubated at room temperature for 5 min, and spun at 1,000 r.p.m.³. After being incubated at 1 °C for 1 h, the mixtures were gently resuspended and tested for rosette formation.

Table 3 E-rosette induction by various enzymes in Daudi cells

Enzymes	Source	Concentrations ($\mu\text{g ml}^{-1}$)	Incubation* min	pH	% Rosettes
Phospholipase A	<i>Naja naja</i>	> 320	30	7.2	> 95
		80	30	7.2	51
	Bee venom†	160–80	30	7.2	3–5
		< 80	30	7.2	0
	Bovine pancreas‡	500–10	30	7.2	0
Neuraminidase§	<i>Vibrio cholerae</i>	100–1 (U ml^{-1})	30	7.0	0
Bromelain	Pineapple	1–0.1 (%)	5	7.2	0
Papain		1–0.1 (mg ml^{-1})	30	7.2	0
Trypsin		0.1–0.01 (%)	30	7.4	0

* Incubation of cells with enzymes other than trypsin (22 °C) were performed at 37 °C.

† Sigma. Activity unit as assayed by PE: 82.7 $\mu\text{mol min}^{-1} \text{mg}^{-1}$.

‡ Boehringer Mannheim. 9.2 $\mu\text{mol min}^{-1} \text{mg}^{-1}$.

§ Worthington.

|| Sigma.

erythrocytes was identical to that of erythrocytes forming rosettes on the PLA-treated Daudi cells.

Several enzymes, including PLA from bee venom and bovine pancreas were also tested for their ability to induce the rosette formation (Table 3). Although bee venom PLA induced rosette formation in a few cells, no other enzymes, including bovine pancreas PLA, could induce a significant rosette formation. It may not be surprising that there was a different effect on the Daudi cells among the three different PLAs. It has been reported that PLAs obtained from these three different sources had different abilities to degrade the phospholipid of human erythrocyte membranes; the erythrocytes were the most sensitive to the *Naja naja* venom PLA, less sensitive to the bee venom PLA and much less sensitive to the bovine pancreas PLA as measured by the degradation of total phospholipid, as well as lecithin, of the cell membranes¹⁴.

Finally we tested the effect of PLA on E rosette formation in the peripheral blood lymphocytes. The unfractionated and B-enriched lymphocyte preparations were incubated at 37 °C for 30 min in the presence or absence of 320 $\mu\text{g ml}^{-1}$ PLA and, after washing, tested for rosette formation by SRBCs. For the enumeration of B cells the detection of receptors for aggregated IgG was carried out (Table 4). The results show clearly that the values for aggregate binding and rosettes after PLA treatment were apparently above 100%, indicating that a population of B cells, if not all

cells, which possessed the receptor for aggregates acquired the rosette-forming property after PLA treatment.

In conclusion, we have shown that treatment of human lymphoblastoid cells as well as a population of B lymphocytes with *Naja naja* venom PLA resulted in exposure of the receptor(s) for E rosette on the cell. The exposure is probably attributable to the degradation of phospholipids of cell membranes that may cover or competitively inhibit the receptor by a steric hindrance. Earlier studies have demonstrated that the displacement of phospholipids from liver and fat cell membranes by PLA can result in the unmasking of substantial quantities of insulin receptor¹⁶.

Regardless of the exposure mechanism, our findings and previous studies on the neuraminidase effect on the E rosette formation¹⁰ suggest strongly the possibility that the receptor for E rosette may be present on the human lymphoid cells independently of T or B populations, although, in the latter, it may exist in a state where the activity is masked or inhibited by membrane constituents such as phospholipid and/or sialic acid.

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Table 4 Effect of PLA treatment on E-rosette formation of peripheral blood lymphocytes

Lymphocytes	Receptor for aggregated IgG‡ %	E-rosette (%)	
		Non-treated	PLA-treated
Unfractionated*			
Case I	28	70	86
Case II	24	73	91
B-enriched†			
Case I	77	15	60
Case II	70	11	65

* Lymphocyte preparations in RPMI-1640 medium containing 10% foetal calf serum were incubated in the Petri dishes at 37 °C for 1 h to remove monocytes. Thus, lymphocyte preparations used were composed of more than 95% small lymphocytes, less than 5% of monocytes (as judged by peroxidase staining) and almost nil neutrophils.

† The partially purified B-cell population was made by E-rosette formation of T cells and separation of the rosettes by Ficoll–Isopaque gradient as described by Wybran *et al.*^{5,15}.

‡ The cells were incubated in the 20 mg ml^{-1} of heat-aggregated IgG, washed twice with GVB and then stained with FITC-conjugated anti-human IgG (γ) sera (Dakopatts).

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Induction of colony formation *in vitro* by human lymphocytes

THE development of a method for colony formation *in vitro* in semi-solid medium by normal macrophages and granulocytes¹⁻³, has been a useful aid in elucidating the control of growth and differentiation of these two types of normal white blood cells and the changes in this control that occur in leukaemia and other diseases⁴⁻⁶. The formation of these two types of colonies requires the addition of an inducing protein, which we call MGI⁵, that is found in the appropriate serum or condition medium. This inducer does not induce the formation of lymphocyte colonies and it would obviously be of value to obtain colony formation *in vitro* by normal bone marrow-derived (B) and thymus-derived (T) lymphocytes.

Although induction of macrophage and granulocyte colonies does not seem to require addition of a foreign antigen, normal lymphocytes require antigenic stimulation for differentiation in mass culture in liquid medium^{7,8}. This suggested that colony formation with normal lymphocytes might be induced in semi-solid medium by providing the appropriate antigenic stimulus. We have now developed a procedure for colony formation *in vitro* with normal human lymphocytes, by stimulating these cells with the lectins phytohaemagglutinin (PHA) or pokeweed mitogen (PWM). PHA has been reported to be mitogenic for T

lymphocytes and PWM mitogenic for both T and B lymphocytes⁹.

Our experiments were carried out with normal human lymphocytes isolated from peripheral blood after centrifugation on a Ficoll-Hypaque gradient¹⁰. To obtain the appropriate stimulation, 8×10^6 lymphocytes were first incubated with PHA or PWM for 1 d in mass culture in liquid medium in Eagle's medium with 25% autologous human plasma and 10^7 erythrocytes. After washing the cells, 5×10^5 lymphocytes were generally seeded per 35-mm Petri dish for colony formation in soft agar (0.33%) on a harder agar base (0.5%) (ref. 1) in Eagle's medium and 20% autologous human plasma, with the addition of new PHA or PWM to the lower agar layer.

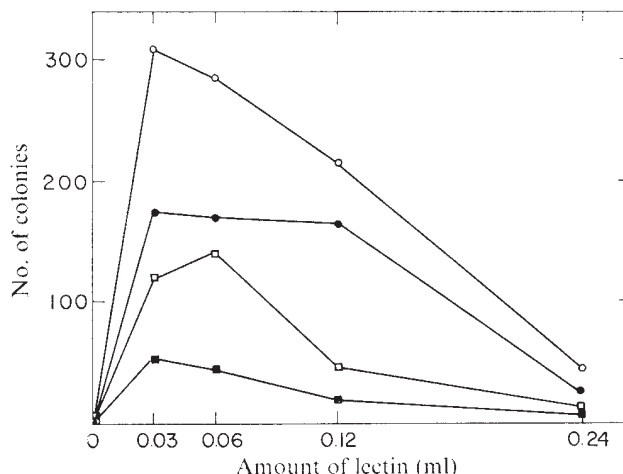


Fig. 2 Number of colonies induced by different concentrations of PHA or PWM. ●, Type I and ○, type II colonies induced by PHA; ■, type I and □, type II colonies induced by PWM. Peripheral blood from healthy adult volunteers was collected with 50 U per ml heparin (pyrogen and preservative free, Evans Medical Ltd). The lymphocytes were isolated by Ficoll-Hypaque gradient centrifugation¹⁰. Cells from the interphase containing more than 95% lymphocytes were washed 3 times with Eagle's medium with a fourfold concentration of amino acids and vitamins (H-21, Grand Island) and 8×10^6 lymphocytes were seeded in 15×95 mm plastic tubes with 6 ml Eagle's medium, 2 ml fresh autologous plasma, 10^7 autologous erythrocytes and 0.1 ml Bactophytohemagglutinin M (PHA) (Difco) or Pokeweed mitogen (PWM) (Grand Island). After 1 d of incubation at 37 °C in this liquid medium, the cells were washed 3 times with Eagle's medium and 5×10^5 lymphocytes were seeded for colony formation in 0.85 ml soft agar (0.33% agar) on a 2.5 ml harder agar base (0.5% agar)¹ per 35-mm Petri dish (No. 1008, Falcon) in Eagle's medium with 20% autologous plasma. Different amounts of PHA or PWM were added to the lower agar layer. Colonies induced by PHA or PWM containing more than 50 cells were counted with an inverted microscope at 5 and 7 d, respectively, after seeding in agar.

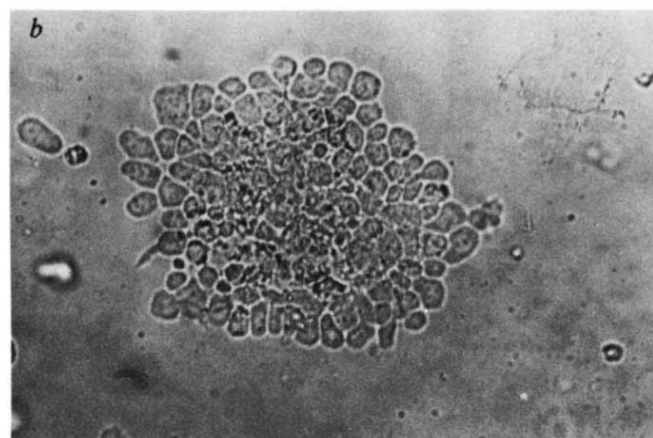
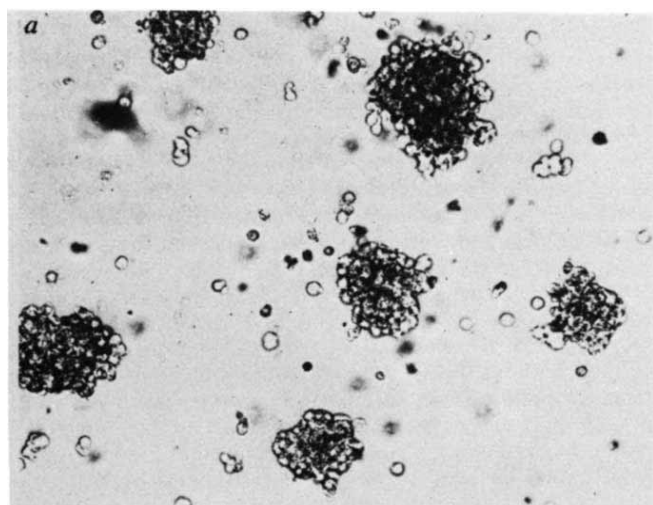


Fig. 1 Photographs of colonies of human lymphocytes in agar induced by PHA. *a*, Type I colonies 4 d after seeding; *b*, type II colony 6 d after seeding. *a* $\times 125$; *b* $\times 270$.

Lymphocytes seeded thus gave rise to two types of colonies: the first, which we will call type I, grew inside the upper agar layer (Fig. 1*a*) and the second, type II (Fig. 1*b*) grew on top of the agar. At 5 d after seeding cells in agar with PHA, type I colonies contained up to about 500 cells and type II colonies up to about 200 cells. With PWM, type I and type II colonies contained up to about 200 cells at 7 d. The colonies induced by both lectins had generally degenerated by 10 d and most of the single cells that did not form colonies had degenerated after 3 d. PHA-induced colonies were counted at 5 d and PWM-induced colonies at 7 d. Only colonies containing more than 50 cells were counted. After incubation for 1 d in liquid medium, the cells were agglutinated by both lectins, but these aggregates were dispersed when the cells were washed and there were no such aggregates after seeding in agar. Transfer of both types of colonies to mass culture in liquid medium at 5 and 7 d after seeding with PHA or PWM, respectively, without

adding new lectin, showed that, in liquid medium, the cells from the colonies did not degenerate for at least another 10 d. Cells seeded for colony formation in 1.2% methylcellulose² (Dow Chemicals) instead of in soft agar, gave only one type of colony, type I, but the total number of colonies was the same in agar and methylcellulose.

Studies with different concentrations of PHA and PWM have shown (Fig. 2) that the number of colonies formed was related to lectin concentration and that the optimum results were obtained with about 0.03 ml lectin per Petri dish. At this concentration, the total number of colonies with cells from different normal donors was 491 ± 98 for PHA and 170 ± 33 for PWM per 5×10^5 cells seeded (Table 1). The number of colonies was related to the number of cells in the range of 5×10^5 cells and there were no colonies when the seeding level was below 10^5 cells per 35-mm Petri dish (Fig. 3). This suggests that colony formation may require a critical concentration of some factor(s) produced by the seeded cells, in addition to the added lectin.

Mercaptoethanol (10^{-4} – 10^{-5} M), which has been reported to increase the viability of normal lymphocytes^{11,12}, did not induce the formation of colonies or enhance the number of colonies induced by PHA or PWM. No colonies were formed when PHA or PWM were omitted either from the preincubation in liquid medium or from the assay for colony formation, indicating that induction of colony formation required the continued presence of the lectin. There were also no colonies, even in the continued presence of the lectin, when autologous human plasma was replaced by foetal calf serum (FCS) in both the preincubation and the colony-forming assay. Substitution of FCS for human plasma only in the assay for colonies, gave at least a 75% decrease in the number of colonies.

Staining of cells from the colonies has shown that both types of colonies induced by PHA or PWM were lymphoblasts, with a similar appearance to the lymphoblasts found after incubation with these lectins in liquid medium. These lymphoblasts did not show phagocytosis of agar. The present procedure did not induce the formation of macrophage, granulocyte¹⁻³ or erythroid¹³ colonies. The formation of lymphoblast colonies by PHA has also been obtained in other experiments with lymphocytes from bone marrow, spleen or peripheral blood¹⁴.

Cells from the lymphoblast colonies in agar were tested for their ability to form rosettes with sheep erythrocytes (E rosettes) that are formed with human T lymphocytes¹⁵⁻¹⁷ and with sheep erythrocytes coated with antibody (EA

Table 1 Number of colonies with peripheral blood lymphocytes from five different normal donors

Donor no.	PHA		PWM	
	No. of colonies Type I	Type II	No. of colonies Type I	Type II
1	188	320	55	102
2	131	199	24	90
3	211	423	45	127
4	165	350	66	140
5	155	312	71	129
Mean $\pm$ s.d.	170 $\pm$ 28	321 $\pm$ 72	52 $\pm$ 17	118 $\pm$ 18

The cells were incubated in liquid medium with 0.1 ml lectin and seeded in agar with 0.03 ml lectin. The number of colonies are per 5×10^5 cells seeded. In the cultures in liquid medium, there were $71 \pm 10\%$ blast cells with PHA and $53 \pm 7\%$ blast cells with PWM, 3 d after seeding.

rosettes) or with antibody and complement (EAC rosettes) that are formed with B lymphocytes¹⁸. Since cells from isolated colonies were surrounded by considerable amounts of agar even after washing, pooled colonies of both types were incubated overnight in liquid medium, washed and then tested for their ability to form rosettes. Many of the cells from PHA-induced colonies formed aggregates in the rosette assays, but none of the PWM-induced colonies did so. In the assay for E rosettes, there were also some erythrocyte aggregates in the incubations with cells from PHA-induced colonies, but not from PWM-induced colonies. The quantitation of rosette-forming cells was, therefore, more reliable with the cells from PWM-induced colonies. They contained about 60% cells with E and about 25% cells with EA and EAC rosettes. About 10% of the cells were stained for surface immunoglobulin (Ig) by fluorescent anti-human IgG or the Fab fragment of IgG (Miles-Yeda). These results indicate that the PWM-induced colonies formed *in vitro* contained T and B lymphocytes. The PHA-induced colonies contained 60% cells with E rosettes and showed no staining for surface immunoglobulin.

These results, showing *in vitro* colony formation with normal human lymphocytes, should be of value for studies on antigenicity, the immune response and the results of virus infection and lymphocyte diseases. Our finding of optimum colony formation with human plasma should also be of value for studies on the role of plasma factors in the control of growth and differentiation of lymphocytes.

Since these experiments were completed, it has been reported¹⁹ that colonies of lymphoid cells with the B-cell markers, EA rosettes and fluorescent staining for surface Ig but without EAC rosettes, were obtained by adding mercaptoethanol to cultures of mouse haematopoietic organs. We did not obtain colonies by adding mercaptoethanol to isolated normal human peripheral blood lymphocytes, and in our experiments, stimulation with the appropriate lectin produced colonies containing cells with markers for T and B cells.

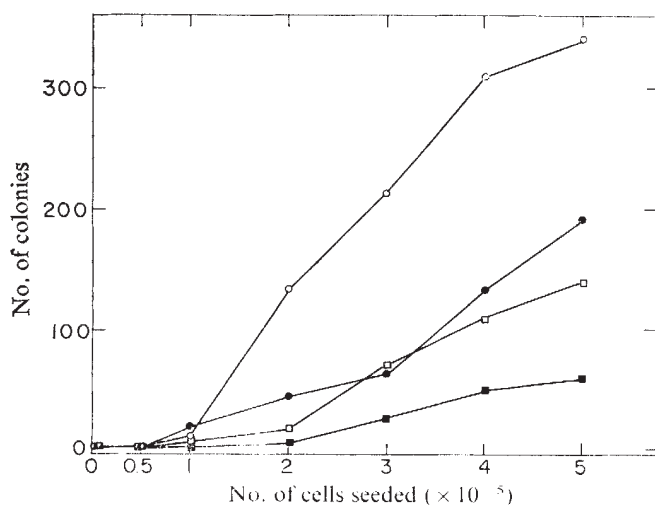
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Fig. 3 Relationship between number of colonies and number of cells seeded. ●, Type I and ○, type II colonies induced by PHA; ■, type I and □, type II colonies induced by PWM. Cells were incubated for 1 d in liquid medium with 0.1 ml lectin and then seeded for colony formation with 0.03 ml lectin.



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Colony formation *in vitro* by leukaemic cells in acute myelogenous leukaemia with phytohaemagglutinin as stimulating factor

HUMAN bone marrow contains cells which form leukocyte colonies of 50 or more cells. Each such colony arises from a single cell¹, belonging predominantly to the myeloid series^{2,3}, which is subject to regulation *in vitro* by humoral colony-stimulating factor generated by peripheral leukocytes^{4,5}.

Bone marrow cells in untreated acute myelogenous leukaemia (AML) show abnormal growth *in vitro* in the Robinson assay⁶. Characteristically, there is near total failure of colony formation; predominantly clusters are formed containing 20 cells or less^{7,8}. Because such poor proliferation *in vitro* might not represent maximum *in vitro* and *in vivo* proliferation of the leukaemic cells, we have studied several modifications of the *in vitro* culture technique. We report here an *in vitro* system in which marrow cells from untreated AML and AML in relapse were stimulated by phytohaemagglutinin (PHA) to form leukaemic cell colonies in soft agar.

Basically, the PHA assay consists of two phases; an initial liquid phase of 15 h at 37 °C and a semi-solid period of 7 d incubation at 37 °C. In the liquid phase 2×10^6 marrow cells per ml of medium (Dulbecco's minimal essential medium + 10% foetal calf serum (FCS) + 10% human serum (HS)) were cultured in Pyrex glass tubes to which 0.05 ml PHA (Difco, PHA-M) per ml medium was added. Bone marrow cells were obtained by aspiration from the iliac crest. The technique used for preparing a marrow cell suspension suitable for tissue culture has been described before⁹. After 15 h of incubation, the cells were washed twice using Hanks' balanced salt solution (305 mosM) and resuspended in agar medium (final concentration agar 0.25%, medium Dulbecco's MEM + 10% FCS + 10% HS). After being resuspended in 0.2 ml agar medium, 1×10^5 cells were pipetted into 35-mm Falcon Petri dishes containing 1 ml agar medium (final concentration agar 0.5%, medium Dulbecco's MEM + 10% FCS + 10% HS) to which 1×10^6 peripheral blood leukocytes from normal individuals were added using the method of Robinson¹⁰. Simultaneously, 1×10^5 cells were plated in Petri dishes containing agar underlayers without leukocytes. After 7 d of incubation in a 7.5% CO₂ gas-controlled humidified incubator at 37 °C, colonies were visible microscopically. Using an inverted

microscope, the colonies were counted. Aggregates containing 50 cells or more were considered colonies.

Using this PHA assay, we tested the growth of marrow cells from both untreated AML patients and AML patients in relapse. In both cases, the blast cell concentration in the bone marrow exceeded 70%. Moreover, in simultaneous experiments, marrow was tested from leukaemic patients in remission and from haematologically normal individuals. Table 1 summarises the results and shows that *in vitro* colony formation of the marrow from 14 untreated AML patients and from four AML patients in relapse did not occur in the conventional Robinson system. In contrast, colonies were observed in the *in vitro* PHA assay. It seems

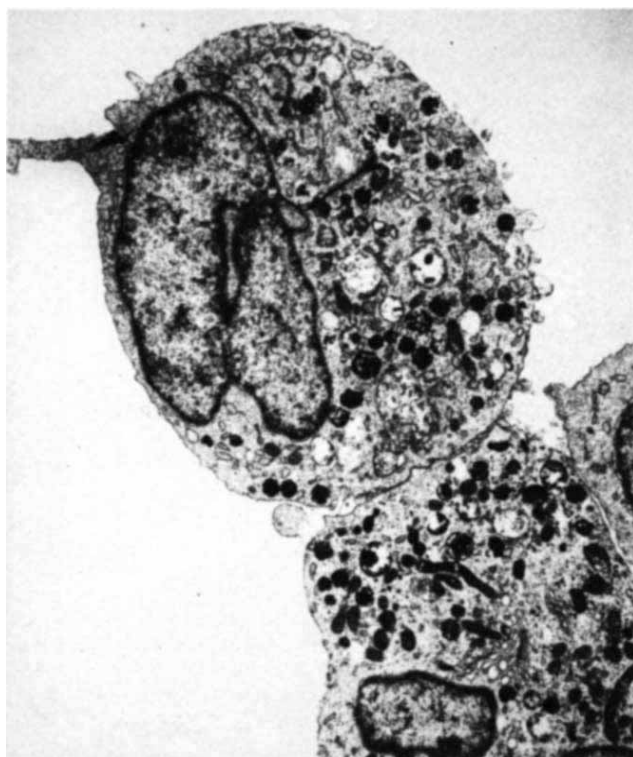


Fig. 1 Ultrathin cross section of a cell in a 7-d-old colony in the PHA assay from marrow of a patient with AML relapse ($\times 6,750$). The nuclear pocket (arrow) and the peroxidase-positive granules are clearly visible. The nuclear pocket is consistent with chromosome abnormalities in the leukaemic cell population¹³.

from the results in Table 1, column 5 that the presence of leukocytes was not essential for colony formation by leukaemic marrow. This contradicts the data of Metcalf *et al.* and Moore *et al.*, who observed no dependence of the presence of leukocytes *in vitro* for leukaemic cell proliferation^{11,12}. An explanation for the discrepancy between our results and the Australian data might be that PHA stimulates a different cell sub-population of the leukaemic pool.

Table 1 Mean number of marrow colonies from AML patients and haematologically normal individuals in the Robinson assay and the PHA assay

Bone marrow	No. of patients	Leukocyte feeder*	Leukocyte feeder	Agar†
Untreated AML	14	0 (0)	99 (14–290)	113 (22–440)
Relapse AML	4	0.5 (0–2)	125 (52–160)	90 (0–130)
Remission AML	10	22 (5–44)	16 (8–46)	0.16 (0–1)
Haematologically normal	5	34 (25–44)	29 (22–35)	0 (0)

*Mean number of CFU-C per 10^5 marrow cells plated. Figures in parentheses represent the range of colonies obtained.

†Cells plated on agar underlayers without leukocyte feeder.

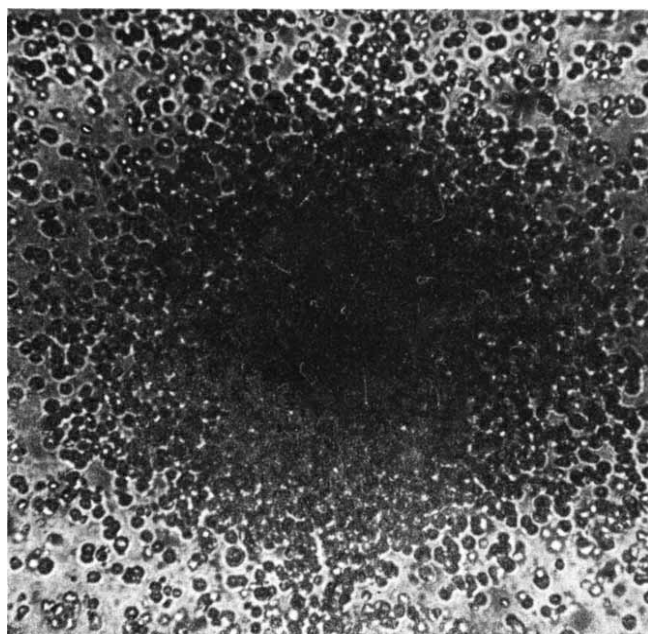
The relationship between the number of cells plated and the number of colonies obtained which was determined in one patient, was linear. This and additional dose-response curves to confirm this observation will be presented later.

Table 1 shows that bone marrow cells in the remission phase of AML form colonies in the Robinson assay as well as in the PHA assay provided that leukocytes are present. The number of colonies scored in the Robinson system is equivalent to that in the PHA assay, indicating no additional effect of PHA on colony formation by marrow cells in remission. Moreover, these data clearly demonstrate the absolute dependence on the leukocyte factor for colony formation by the remission marrow. The same dependence prevails for colony formation of marrow cells from haematologically normal individuals. The similarity of *in vitro* growth between remission marrow and haematologically normal marrow is further evidence for their identity.

Evidence for the leukaemic origin of the bone marrow colonies from untreated AML patients and from AML patients in relapse was obtained by chromosome analysis of cells in the colonies and from morphological studies at the ultrastructural level. These detailed studies will be published soon. Figure 1 shows an electron micrograph of a cell in a colony from the bone marrow of an untreated AML. The nuclear pocket or bleb is apparent on the surface of the nucleus. This morphological alteration is consistently observed in leukaemic cells demonstrating an abnormal chromosome pattern¹³. The karyotype of the leukaemic cell population in this patient was 45,X,-Y,-C,+D,+E,-G, a complex pattern which has been observed in other acute leukaemic patients¹⁴. Figure 2 shows a colony obtained from the bone marrow of an untreated AML patient. This particular colony consists of approximately 500 cells.

This is a first report of PHA-induced mitogenesis of human leukaemic cells *in vitro*, which is distinct from agglutination of malignant cells by lectins^{15,16}. The cultures were checked for cell agglutination immediately after plating. Although in two cases clumps of 2-10 cells were present, in all other cases bone marrow suspensions were distributed in agar as single cells. For this reason further research in technology is needed before an exact assessment can be made of PHA stimulation in every leukaemia.

Fig. 2 Colony cells (7 d old) from an untreated AML in the PHA assay. In this particular colony, 500-700 cells are present ($\times 45$). The leukaemic origin of the colony has been established by electron microscopy.



Aggregation by cellular migration after culture was excluded by counting the number of aggregates in the PHA assay and by comparing that with the number of aggregates in the Robinson system, which was done in eight cases. In all eight cases the total number and size of aggregates in the PHA assay exceeded those in the Robinson assay, which suggests that PHA induced greater cellular proliferation than occurs in the Robinson assay.

Although the mode of action of PHA on the leukaemic cell population is not known, PHA seems to stimulate leukaemic cells to form colonies. But cell types other than leukaemic cells may survive in the PHA *in vitro* cultures. In spite of this, the *in vitro* phenomenon described here demonstrates the growth potential of a leukaemic cell population. It is very important to study whether or not the leukaemic stem cell *in vivo* belongs to the leukaemic subpopulation which is sensitive to PHA *in vitro*. Meanwhile, this *in vitro* phenomenon may be useful in the detection of residual leukaemic cells during the remission phase of AML.

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Development of colonies *in vitro* of mitogen-stimulated mouse T lymphocytes

TECHNIQUES have been developed for the growth of haemopoietic cells (see refs 1 and 2), human T lymphocytes³ and mouse B lymphocytes⁴ on soft agar. Experiments with mouse lymphocytes suggest that concanavalin A (con A) and phytohaemagglutinin (PHA) stimulate T cells^{5,6}, whereas pokeweed induces proliferation of both B and T cells⁷. Based on previously described techniques for obtaining clonal growth of human lymphocyte colonies *in vitro*³, a procedure has now been developed for inducing clonal proliferation of mitogen-stimulated mouse lymphocytes using a two-layer soft agar technique.

Cells were obtained by removing inguinal and mesenteric lymph nodes and the spleen, in aseptic conditions, from mice of strains ICR, C3H/HeJ, C57BL/6 and congenitally athymic (*nu/nu*) mice.

The organs were crushed, and passed through stainless steel mesh into cold phosphate-buffered saline. Cell suspensions in Eagle's medium (EM) were cultured at 37 °C in a water-saturated atmosphere with 5-7.5% CO₂ for 48 h in a concentration of 5 × 10⁶ per ml EM containing PHA-M (Difco, 0.0125 ml ml⁻¹), or con A (Difco, 30 µg ml⁻¹), and 5% fresh normal human pooled serum which had been heat inactivated at 56 °C for 30 min. The

mitogen-stimulated lymphocytes were washed twice in EM and the resuspended cells were seeded in the upper agar layer, 10^6 cells to each 30-mm Petri dish. The lower agar layer consisted of 2.5 ml medium containing modified Eagle's medium, 20% heat-inactivated human serum, 0.5% agar and $0.0125 \text{ ml ml}^{-1}$ PHA or $30 \mu\text{g ml}^{-1}$ con A.

The upper layer (0.85 ml) consisted of EM, 20% inactivated human serum, 0.32% agar, and the seeded lymphoid cells. This was incubated at 37°C in a humidified atmosphere with 5–7.5% CO_2 . As controls, cultures were set up in which the lower agar layer did not contain mitogen, or the lymphoid cells present in the upper layer had not been sensitised with PHA or con A.

The development of colonies and their morphology were observed under an inverted microscope at $\times 50$. The majority of seeded cells underwent lysis during the first and second days of culture, while the surviving cells proliferated and developed into colonies. Through days 3–5 of culture, each colony was made up of at least 50 cells, with most colonies reaching larger size and containing about 1,000 cells. Following a stationary period of 2–3 d the colonies began to degenerate on day 7 or day 8, and on day 11 the colonies showed complete disintegration with lysis of the cells.

Studies of the cell morphology of the colonies which appeared after 3–4 d, showed all cells to be large and pyroninophilic, resembling in their ultrastructure the blast-like cells which are observed on culturing lymphoid cells in a liquid medium in the presence of PHA or con A. A mitotic index of 5–6% was determined in colony cells after 5 d on soft agar cultures.

When spleen cells were seeded in addition to lymphocyte colonies, macrophage and granulocyte colonies developed, at the same time or 1–2 d later. Also, in some experiments a new kind of colony, composed of large macrophages, developed after days 12–14 and increased in size to day 20.

Similar to the ability of lymphocytes to produce a factor stimulating the growth of bone marrow macrophages and granulocytes⁸, PHA- or con A-sensitised murine lymphocytes in soft agar cultures produced a colony-stimulating factor which may have stimulated the macrophage colonies.

Fig. 1 Effect of varying the concentrations of PHA in the lower agar layer, and of human pooled heat inactivated serum in the agar layers on development of colonies. ●, 20% human pooled serum; ▲, 10% human pooled serum; ■, 5% human pooled serum.

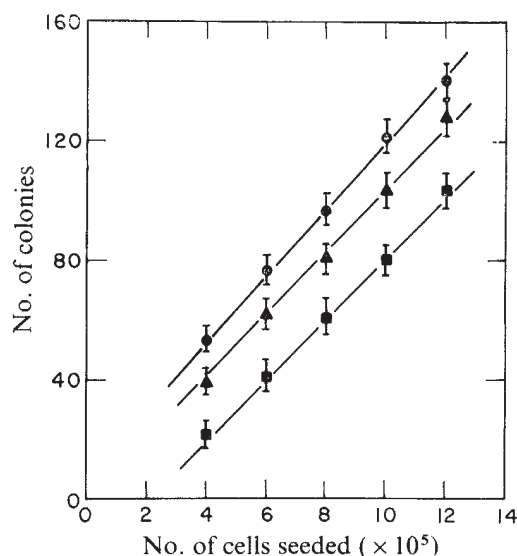
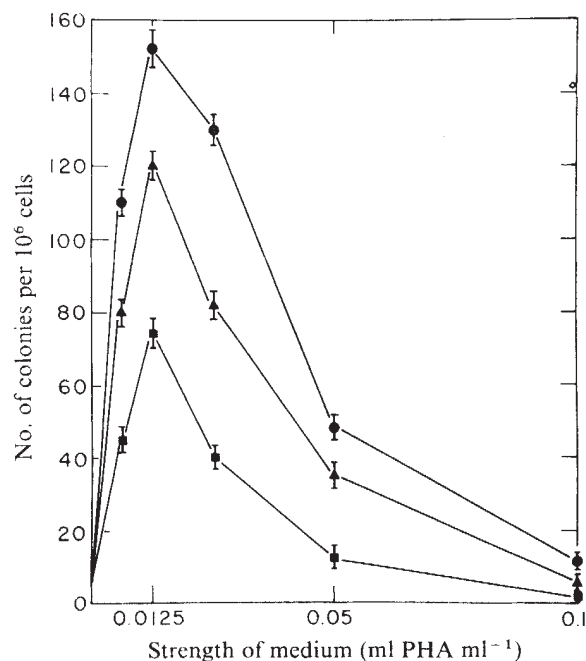


Fig. 2 A linear relationship between the number of cells seeded and the number of resulting colonies with either PHA ($0.0125 \text{ ml ml}^{-1}$) or con A ($30 \mu\text{g ml}^{-1}$) in the culture were used. For the same mouse strains, the number of colonies was greatest when PHA was used as mitogen. ●, PHA, ICR; ▲, PHA, C3H/HeJ; ■, con A, ICR.

The number of lymphocyte colonies developing in specific experiments, depended on the mitogen used, the organ of origin, and other specific conditions. We obtained our best results with 20% inactivated freshly pooled human serum, while 0.0125 ml PHA or $30 \mu\text{g}$ con A per ml medium gave the best cell preservation, and facilitated the development of large numbers of colonies (Fig. 1). An approximately linear relationship was found between concentration of PHA in the agar layer and number of developing colonies for low concentrations; concentrations exceeding $0.0125 \text{ ml ml}^{-1}$ depressed colony formation (Fig. 1).

Presensitisation with the mitogens, as well as their continuous presence in the soft agar, were essential conditions for colony formation. Incubation periods with PHA in liquid medium exceeding 48 h did not cause any further significant increase in the number of developing colonies.

A linear relationship was observed between number of cells seeded and number of colonies developing, regardless of whether different mouse strains or different mitogens were used. The potential for colony formation of PHA, in comparison, was higher than that of con A in the same conditions of culture (Fig. 2).

In spite of the fact that a great number of lymphoid cells were seeded (10^6 cells per plate), the ability of clonal proliferation was limited to a minority of transformed lymphocytes. No lymphocyte colonies developed when con A- or PHA-sensitised lymph node or spleen cells of congenitally athymic (*nu/nu*) mice were cultured (Table 1).

Table 1 The largest number of T-cell colonies were obtained culturing inguinal lymph node cells from different mouse strains using PHA as mitogen

Mouse strain	Mitogen	Lymphocytes prepared from lymph nodes	Number of colonies/ 10^6 cells (mean $\pm$ s.e.)
ICR	PHA	Inguinal	120 ± 7.4
ICR	PHA	Mesenteric	80 ± 8.2
ICR	Con A	Inguinal	80 ± 5
C3H/HeJ	PHA	Inguinal	110 ± 8.2
C57BL/6	PHA	Inguinal	170 ± 16
C57BL/6	PHA	Mesenteric	62 ± 6.4
congenitally athymic (<i>nu/nu</i>)	PHA or Con A	Inguinal or Mesenteric	0

No colonies were produced by culturing congenitally athymic (*nu/nu*) mice cells known to be markedly deficient in functional T cells.

To identify the nature of the colony-forming cells, indirect immunofluorescent staining of the θ isoantigen⁹ was carried out on cells pooled from colonies obtained after 4–5 d of culture of C3H/HeJ lymph node cells. Colonies were removed with capillary tubes, washed three times with Ringer solution containing 5% inactivated human serum, incubated with anti- θ , washed once more with Ringer solution–human serum; bound anti- θ was detected by using fluorescein isothiocyanate conjugated rabbit anti-mouse immunoglobulin. The θ -bearing cells were revealed by appearance of bright ring-like fluorescence. Demonstration of the θ isoantigen in cells of colonies, as well as the failure to obtain cultures from congenitally athymic nude mice, known to be deficient in T cells, indicate that con A- or PHA-stimulated cells capable of developing into colonies in the soft agar system belong to the T-cell series.

This technique for growing and developing mouse T-cell colonies should be valuable for the investigation of lymphoid cells. Its particular advantage is to allow the study of a lymphocyte colony population derived from a particular lymphocyte series possessing the ability to develop into colonies.

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Replacement of serum by hormones permits growth of cells in a defined medium

MOST cell cultures require the addition of serum to synthetic media for their maintenance and growth, and we believe that the primary role of the serum is to provide hormones¹. We have been led to this hypothesis by a series of experiments showing that serum depleted of certain hormones no longer supports growth of cells, unless the medium is supplemented with the hormones that were removed^{2–4}. Clear evidence for the validity of this hypothesis has not yet been obtained because it is difficult to grow cells in the absence of serum. Recently, however, we have succeeded in growing an established rat pituitary cell line, GH₃, in a defined serum-free medium supplemented with physiological concentrations of four hormones together with the iron transport protein, transferrin. Preliminary investigation shows that serum-free medium supplemented with hormone will also support the growth of several other cell lines.

GH₃ is a functional rat pituitary cell line which produces growth hormone and prolactin^{5,6}, and depends on thyroid hormones for growth¹. Initially, the basal medium used in our growth experiments consisted of 8% charcoal-extracted foetal calf serum. At this concentration of serum, the stimulation by triiodothyronine (T₃) of the growth of these cells was readily demonstrated. As the serum concentration was decreased in a stepwise manner, we were able to find which additional hormones were required; eventually it was

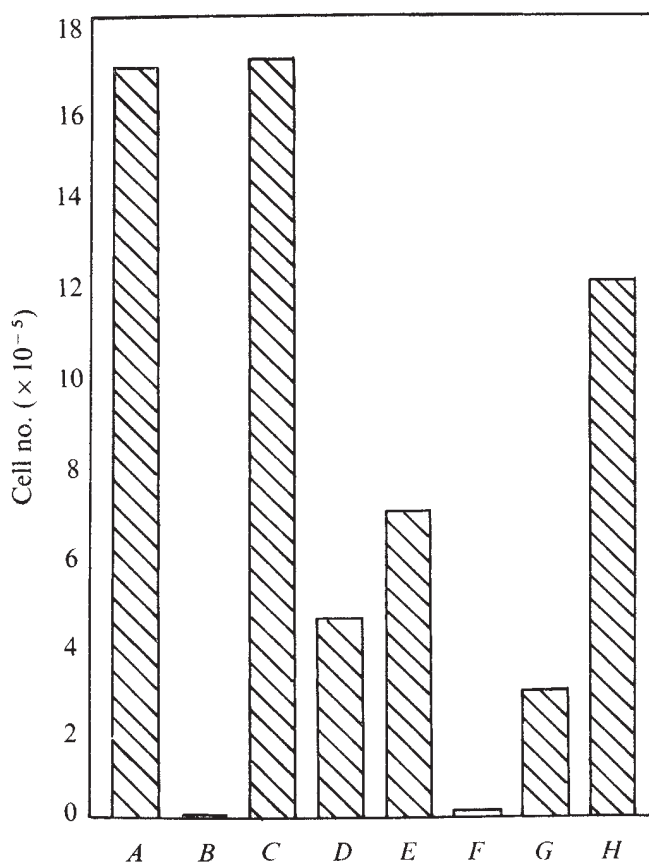
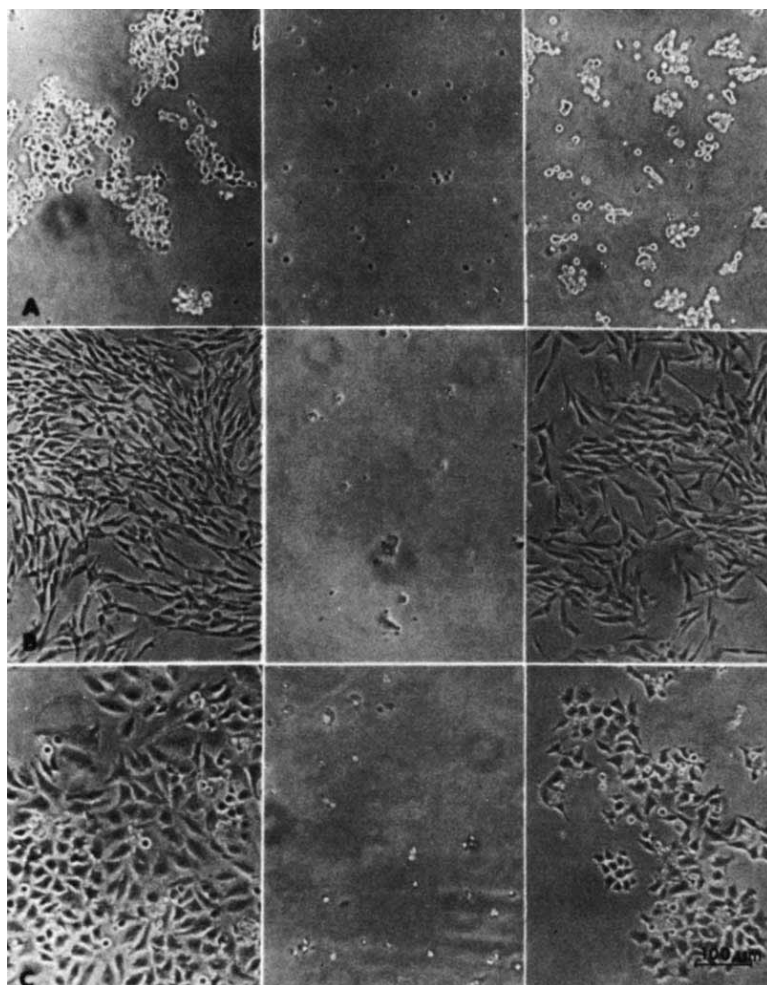


Fig. 1 GH₃ cells after 10 d of culture in F-12 medium supplemented with 8% foetal calf serum (8% FCS F-12), serum-free F-12 medium (F-12 SF), and F-12 SF supplemented with hormones. The cells from stock plates (in Dulbecco's modified Eagle's medium supplemented with 12.5% horse serum and 2.5% foetal calf serum) were trypsinised into single cells, treated with trypsin inhibitor, suspended in F-12 SF, and then inoculated at 1×10^5 cells per 60-mm plate. The hormones were added at the time of plating. The final concentrations of hormones were 3×10^{-10} M T₃, 1×10^{-9} M TRH, 50 mU ml⁻¹ somatomedin preparation, 5 μ g ml⁻¹ transferrin and 0.5 ng ml⁻¹ PTH. At the end of the experiments, both the floating and the attached cells were collected by trypsinisation and centrifugation, treated with Trypan blue, and the live cells counted by haemocytometer. A, 8% FCS F-12; B, F-12 SF; C, F-12 SF+hormones (T₃+TRH+somatomedin A+transferrin+PTH); D, F-12 SF+hormones minus T₃; E, F-12 SF+hormones minus TRH; F, F-12 SF+hormones minus somatomedin A; G, F-12 SF+hormones minus transferrin; H, F-12 SF+hormones minus PTH. The first-day cell counts for A and C were identical (0.9×10^5 cells per plate). T₃ and transferrin (human) were obtained from Sigma, TRH is a synthetic peptide and PTH is the synthetic peptide of the first 30 amino acids of human PTH.

found possible to eliminate serum entirely if the medium was supplemented with T₃, thyrotropin-releasing hormone (TRH), transferrin, the biologically active peptide of parathyroid hormone (PTH), and a partially purified somatomedin preparation (5,000-fold purification from serum)⁷. The effect of these hormones on cell growth is shown in Figs 1 and 2. In the control plates, where neither serum nor hormones were added, there were no live cells after 3 d of incubation. When the five components are present in the serum-free medium, the growth is ~60–100% that in the rich medium (8% foetal calf serum in F-12). If any of the components is not added to the medium, growth either does not occur or is severely depressed. This deficiency is not overcome by substituting other pituitary hormones, steroids, hypothalamic releasing hormones, caeruloplasmin, insulin, glucagon, glycyl-histidyl-lysyl acetate, calcitonin, or prostaglandin E₁.

The results obtained with GH₃ cells encouraged us to

Fig. 2 Three cell lines in various media after 7 d in culture. Cells: A, GH₃; B, BHK; C, HeLa. Media from left to right, 8% FCS F-12, F-12 SF, F-12 SF supplemented with hormones. Hormone additions to GH₃ and BHK are described in the text and in Fig. 3. The final concentrations of hormones added to HeLa cultures were: 1×10^{-9} M TRH, 10 ng ml^{-1} SRIF and LRH, 2×10^{-9} M hydrocortisone acetate, 17β -oestradiol and testosterone, 2×10^{-8} M progesterone, 3×10^{-10} M T₃, $5 \mu\text{g ml}^{-1}$ caeruloplasmin and transferrin, $0.2 \mu\text{g ml}^{-1}$ glycyl-histidyl-lysyl acetate, 50 mU ml^{-1} somatomedin A, 50 ng ml^{-1} insulin and glucagon 0.5 ng ml^{-1} PTH and 0.1 ng ml^{-1} calcitonin.



study the growth of other established cell culture lines in a defined serum-free medium supplemented with hormones. Initial experiments showed that BHK and HeLa cells do not survive in serum-free medium, but can grow in serum-free medium supplemented with a mixture of 25 hormones. The growth in the latter medium is comparable to that in the rich medium. The growth response of these cells to hormones is shown in Figs 2 and 3. Determinations of the specific hormones which support the growth of these cell lines is in progress.

There have been several reports of the growth of cells in completely defined medium⁸⁻¹⁰. Most if not all of these cases are, however, examples of selection or adaptation of cells to defined culture media. By contrast, our data show that the medium supplemented with hormone substitutes for serum without altering the characteristics of the individual cells or the overall population. Our experiments indicate that a combination of hormones and other specific factors, such as transferrin, can substitute completely for serum. Though the possibility of serial propagation of GH₃ is still under investigation, the results obtained with BHK (Fig. 3) strongly suggest that serum-free medium supplemented with hormones will also support the long term growth of GH₃ cells in a completely defined medium. It has already been shown that hormones can partially substitute for some functions of serum such as the overcoming of density-inhibited growth on changing from serum-containing to serum-free medium¹¹.

In these experiments, there is a possibility that residual serum factors are still present and active even after extensive washing. We eliminate this possibility in our experiments in which the cells are transferred from serum-containing medium by trypsinisation into defined medium,

and the growth studied entirely in the defined, serum-free medium. It is important to note that there is no delay in the initiation of growth, suggesting that there is neither selection of cells capable of growth in the serum-free medium, nor extensive adaptation to the growth conditions. Furthermore, growth, in the case of GH₃ cells, is not a nonspecific response to the addition of protein to the serum-free medium. The total amount of protein added (transferrin and the somatomedin preparation) is about $8 \mu\text{g ml}^{-1}$. Serum protein equivalent to this protein concentration (0.016% foetal calf serum), when added to the serum-free medium, does not support the growth of these cells, even when supplemented with T₃. We attribute the growth-promoting activity of the transferrin and somatomedin preparations to these two specific factors, although we recognise that other components within these preparations might also be responsible for stimulation of growth. We are continuing to investigate this as more highly purified preparations become available.

The results presented here strongly suggest that it is possible to eliminate serum from culture medium, and that the main function of serum in cell culture is to furnish hormones. We expect that any cell culture line showing a requirement for serum can be grown in synthetic medium supplemented with a combination of hormones and a few factors such as transferrin. Comparison of the hormonal requirements of GH₃, BHK and HeLa already indicates that the specific hormones will vary with the cell type, although a few hormones may be common to all cell types. We believe that the elucidation of these requirements will be useful in advancing our understanding of integrated physiology. The possibility of culturing cells in serum-free medium supplemented with hormones will enhance the importance

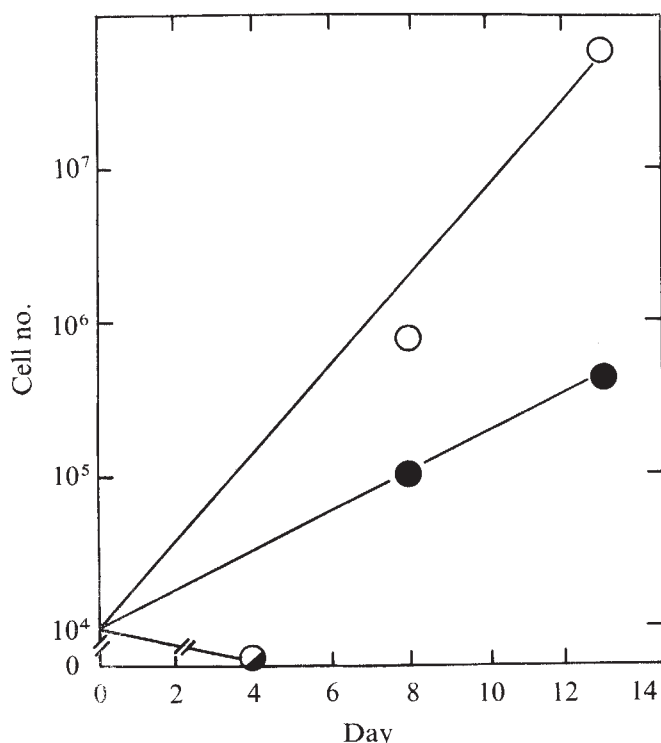


Fig. 3 Growth of BHK cells in 10% FCS F-12 and in F-12 SF supplemented with hormones. There were no live cells in F-12 SF only, 4 d after the initiation of culture. The initial plating procedure is as described in Fig. 1. The cells were inoculated at 1×10^4 cells per 35-mm plate, and the medium was changed on day 5. The cells, both in the rich medium and in the serum-free medium supplemented with hormones were subcultured on day 8 into the respective media. For subcultures, both trypsin and trypsin inhibitor were also used. On day 8 and on day 13 the cells were washed with buffer, trypsinised, and counted with a Coulter counter. The final concentrations of hormones and factors used were: $0.5 \mu\text{g ml}^{-1}$ LH, 10 mU ml^{-1} FSH, $5 \mu\text{U ml}^{-1}$ ACTH, 5 ng ml^{-1} GH, 10 ng ml^{-1} prolactin, 5 ng ml^{-1} TSH, 10 ng ml^{-1} prostaglandin E_1 , $5 \mu\text{g ml}^{-1}$ caeruloplasmin and transferrin, $0.2 \mu\text{g ml}^{-1}$ glycyl-histidyl-lysyl acetate, 50 mU ml^{-1} somatomedin A, 50 ng ml^{-1} insulin and glucagon, 0.5 ng ml^{-1} PTH, 0.1 ng ml^{-1} calcitonin, $1 \times 10^{-9} \text{ M}$ TRH, 10 ng ml^{-1} SRIF (somatotropin releasing inhibitory factor) and LRH (LH releasing hormone). ○, 8% FCS F-12; ●, F-12 SF; ●, F-12 SF supplemented with hormones. LH, FSH, GLH, prolactin (bovine) and TSH were supplied by NIAMD Program of National Institutes of Health; ACTH was obtained from Armour Pharmaceutical Co., Chicago Illinois; caeruloplasmin, transferrin and insulin were from Sigma (Bovine) and glycyl-histidyl-lysyl acetate and glucagon were from Calbiochem. Prostaglandin E was from Dr J. Pike, Upjohn Co.

of cell culture as an experimental tool, especially in overcoming the present difficulties associated with obtaining primary cultures.

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Interaction of multiplication-stimulating activity with chick embryo fibroblasts demonstrates a growth receptor

MULTIPLICATION-STIMULATING activity (MSA), which is the name given to a family of polypeptides isolated from calf serum¹ and from medium conditioned by certain rat liver cell cultures², stimulates cellular DNA synthesis and growth, and has insulin-like activity. MSA is acid soluble, heat stable and has a molecular weight of approximately 10,000^{1,2}. It shares these physical and biological properties with somatomedin³ and acid-ethanol soluble non-suppressible insulin-like activity (NSILA-s)⁴—polypeptides in human serum which may be important in human growth. We have now studied the interaction of MSA and insulin with chick embryo fibroblasts (CEF). We describe here the binding of labelled MSA to CEF, and correlate this binding with the stimulation of incorporation of ³H-thymidine into DNA. We propose that MSA and insulin stimulate DNA synthesis in CEF by interacting with a common growth receptor.

MSA was purified from medium conditioned by a cloned line of rat liver cells, BRL-3A², originated by Dr H. Coon. The medium was clarified by centrifugation, chromatographed on Dowex 50 (ref. 2) and twice gel-filtered on Sephadex G-50 in 1 N acetic acid. Fractions which gave a single protein band on disc acrylamide gel electrophoresis at pH 2.7 in 9.0 M urea were used (unpublished results of S.P.N., J. Passamani and P. Short).

Both MSA and insulin⁵ stimulated the incorporation of ³H-thymidine into DNA in tertiary cultures of CEF. The dose-response curves of the two polypeptides were essentially indistinguishable, as were the maximum responses (Fig. 1). Half-maximal stimulation was observed with MSA at 30 ng ml^{-1} and insulin at 45 ng ml^{-1} . Similar results were reported by Smith and Temin⁷.

When CEF were incubated concurrently with MSA at $0.5 \mu\text{g ml}^{-1}$, and insulin at $0.5 \mu\text{g ml}^{-1}$ —concentrations which separately gave maximum stimulation of ³H-thymidine incorporation—the stimulation observed was not

Table 1 Effect of combined addition of MSA and insulin on ³H-thymidine incorporation in CEF

Addition	³ H-thymidine incorporation (c.p.m. per dish $\times 10^{-3}$)
(1) Control	5.7 ± 0.1
(2) MSA, $0.5 \mu\text{g ml}^{-1}$	21.0 ± 0.1
(3) MSA, $1.0 \mu\text{g ml}^{-1}$	19.8 ± 1.4
(4) Insulin, $0.5 \mu\text{g ml}^{-1}$	14.5 ± 1.2
(5) Insulin, $1.0 \mu\text{g ml}^{-1}$	17.2 ± 0.3
(6) MSA, $0.5 \mu\text{g ml}^{-1}$ + Insulin, $0.5 \mu\text{g ml}^{-1}$	$15.9 \pm 0.2^*$
(7) 10% Serum	58.9 ± 2.7

CEF were incubated for 12 h with the additions shown, pulsed for 1 h with ³H-thymidine, and the radioactivity incorporated into DNA was determined. The mean and range of duplicate determinations are shown. The data obtained with insulin and MSA ($0.5 \mu\text{g ml}^{-1}$ and $1.0 \mu\text{g ml}^{-1}$) confirmed that $0.5 \mu\text{g ml}^{-1}$ was a maximally effective concentration.

*If the effects of MSA and insulin were additive, the incorporation expected would be 29.8.

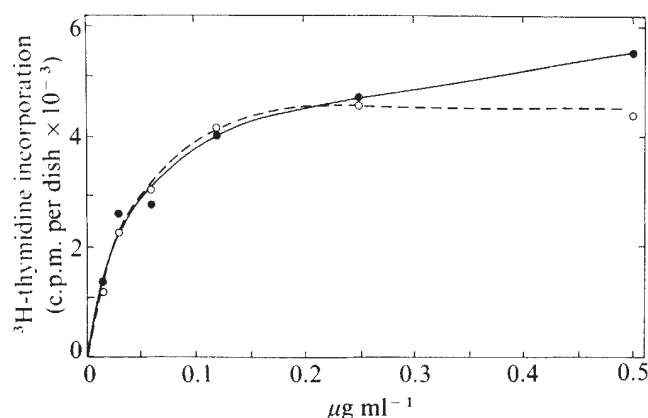


Fig. 1 Stimulation of ^3H -thymidine incorporation in CEF by MSA and insulin. Tertiary cultures of CEF were plated in 0.4% ET (Temin's modified Eagle's medium with 20% by volume tryptose phosphate broth and 0.4% foetal calf serum)². Three days later, insulin (crystalline porcine, Eli Lilly), MSA or ET medium were added at the concentrations indicated. After incubation for 12 h, the cultures were pulsed for 1 h with ^3H -thymidine, $0.2 \mu\text{Ci ml}^{-1}$, and the incorporation of radioactivity into acid-precipitable material (DNA) was determined⁶. The c.p.m. incorporated per 60-mm Petri dish are plotted. The points are the average of duplicate determinations. The incorporation, 2,830 c.p.m., in a control dish which received medium alone, has been subtracted. The MSA and insulin dose-response curves presented are representative. In general, however, the maximal stimulation by MSA (○) is slightly greater than that obtained with insulin (●).

the sum of individual stimulations (Table 1). Since foetal calf serum induced a greater rate of ^3H -thymidine incorporation, the non-additive effects of insulin and MSA suggested that the two peptides act by a common mechanism. Non-additivity has been observed with insulin and NSILA-s⁴, and insulin and MSA (unpublished results) in human fibroblasts, and with insulin and MSA in 3T3 mouse fibroblasts⁸. In contrast, insulin, MSA and NSILA-s gave

additive responses with serum and fibroblast growth factor in these systems^{8,9}.

Polypeptide hormones such as insulin and MSA exert their biological effects by interacting with specific plasma membrane receptors¹⁰. The apparent common pathway for the stimulation of DNA synthesis in CEF by MSA and insulin could be explained if they utilised the same cell surface receptor. To examine this possibility, MSA was radioactivated by the chloramine T procedure as modified by Megyesi *et al.*¹¹ and the binding of ^{125}I -MSA to CEF studied.

^{125}I -MSA bound rapidly and reversibly to CEF at 22°C . Half the maximal binding was achieved in 45 min, and a plateau of binding was maintained for 3–6 h. Approximately 1–4% of the tracer MSA was bound per 10^6 CEF. The bound radioactivity was reduced by more than 70% when incubation was conducted in the presence of an excess of unlabelled MSA ($1 \mu\text{g ml}^{-1}$). Competitive binding with different concentrations of unlabelled MSA gave a steep displacement curve (Fig. 2a). Binding was inhibited 50% by MSA at 50 ng ml^{-1} in this experiment, and at $33 \pm 5 \text{ ng ml}^{-1}$ in 13 experiments. Unrelated peptides failed to inhibit the binding of MSA tracer even in high concentrations: human growth hormone, glucagon and nerve growth factor (A. Liuzzi) at $10 \mu\text{g ml}^{-1}$; epidermal growth factor (R. Ledda) at 600 ng ml^{-1} ; and fibroblast growth factor (D. Gospodarowicz) at 200 ng ml^{-1} . In contrast, in seven experiments insulin was $66 \pm 13\%$ as potent as MSA by weight in competing for MSA tracer binding (Fig. 2a).

The near equivalence of MSA and insulin in competing for the binding of MSA to CEF differs strikingly from their relative potencies in other binding systems. In rat liver membranes, for example, MSA is more than four orders of magnitude more effective than insulin in competing for ^{125}I -MSA binding (Fig. 2b). In cultured human lymphocytes, on the other hand, insulin competes for the binding of MSA tracer appreciably better than does MSA itself (Fig. 2c). This suggests that lymphocytes lack an MSA

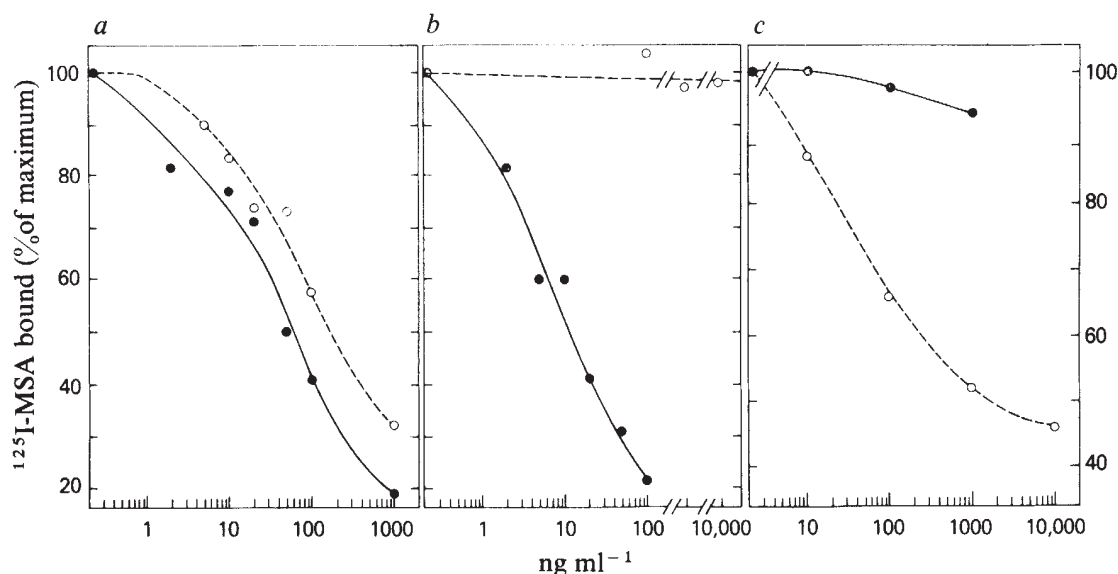


Fig. 2 Binding of ^{125}I -MSA to CEF rat liver membranes and cultured human lymphocytes. MSA was iodinated with Na^{125}I to a specific activity of 150 Ci g^{-1} by a modification of the chloramine T procedure¹¹. ^{125}I -MSA gave a single sharp peak on Sephadex G-50 gel filtration in 1 N acetic acid and co-migrated with unlabelled MSA on disc acrylamide electrophoresis. Tertiary cultures of CEF were detached from the cell monolayer by gentle trypsinisation (0.05% trypsin 0.5 mM EDTA, 37°C , 5 min). Binding experiments were performed under conditions chosen to give a steady state. *a*, ^{125}I -MSA, 500 pg ml^{-1} , was incubated in HEPES binding buffer¹², pH 8.0, in a total volume of 0.5 ml , with 2×10^6 CEF for 3 h at 22°C ; *b*, ^{125}I -MSA (500 pg ml^{-1}) was incubated with purified rat liver plasma membranes (kindly given by D. M. Neville Jr) in 0.15 ml of Krebs-Ringer phosphate buffer, pH 7.4, containing 1% bovine serum albumin for 90 min at 22°C ; *c*, ^{125}I -MSA (500 pg ml^{-1}) was incubated with 8×10^6 cultured human lymphocytes (line IM-9) in 0.5 ml of HEPES binding buffer, pH 8.0, for 90 min at 15°C . The indicated concentrations of unlabelled MSA (●) and insulin (○) were added together with tracer MSA and were present throughout the incubation. In each experiment, cells or membranes are pelleted by centrifugation for 1 min in a Beckman Microfuge, the pellets were excised and the bound radioactivity was determined. The total binding was 48% , 3.7% and 6.2% of the tracer radioactivity in the three experiments, respectively. Maximum binding has been plotted at 100% .

receptor, tracer MSA binding exclusively to lymphocyte insulin receptors. The poor competition by unlabelled MSA is consistent with its low insulin-like activity (<1% the potency of insulin). Analogous results have been reported for NSILA-s binding to rat liver membranes and cultured lymphocytes¹¹.

CEF have insulin receptors (our unpublished results) similar to those in other tissues^{13,14}. The MSA binding we have observed, however, clearly is not binding to an insulin receptor. ¹²⁵I-insulin binding to insulin receptors is inhibited by different peptides in proportion to their insulin-like biological activities. MSA and NSILA-s, which have low insulin-like activity, compete poorly for the binding of insulin tracer. In contrast, they are more than 250 times more potent competitors for MSA binding than for insulin binding in CEF (our unpublished results). Porcine proinsulin is 20 times more potent in competing for MSA binding than for insulin binding (our unpublished results). Scatchard plots of insulin binding data are characteristically curved¹⁵. This results from negatively cooperative site-site interactions among insulin receptors¹⁵. In contrast, Scatchard plots of MSA-binding data are linear (our unpublished results). We have been unable to demonstrate cooperative interactions among MSA receptors.

In summary, CEF possess MSA receptors with a unique specificity, that is, insulin competes almost as well for tracer MSA binding as MSA itself. The CEF MSA receptors differ from insulin receptors, MSA receptors of rat liver membranes, and NSILA-s receptors in CEF¹⁶. MSA and insulin stimulate DNA synthesis in CEF with nearly identical dose-response curves. Their effects are not additive. The same MSA concentration induces half-maximal ³H-thymidine incorporation and inhibits tracer MSA binding by 50%. MSA preparations at different stages of purification and inactivated MSA compete for tracer MSA binding in proportion to their potencies in stimulating ³H-thymidine incorporation (our manuscript in preparation). Taken together, these properties suggest that the MSA receptor we have demonstrated in CEF is a physiologically relevant growth receptor, mediating the growth-promoting effects of MSA and insulin.

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Two sex steroid receptors in mouse fibroblasts in culture

WE report that the mouse fibroblasts known as L-929 cells have androgen and oestrogen receptors (R-A and R-E), as well as the glucocorticosteroid receptors (R-G) reported earlier^{1,2}. This observation may lead to an increased interest in L cells as they provide a convenient system for studies of the biology and pharmacology of hormones at the cellular level.

L-929 cells provided by J. Zeuthen, Institute for Human Genetics, Aarhus, who obtained them from the American Type Culture Collection, and found that they had 56-76 chromosomes, with a modal number of 66 (personal communication). Cells were grown in plastic Petri dishes, or in suspension using Dulbecco's modified Eagle's medium supplemented with 10% heat inactivated calf serum, bovine insulin (0.12 U ml⁻¹) and antibiotics. At confluence, cells were collected by scraping the dishes with a rubber policeman, or by low speed centrifugation from suspension. After washing in cold phosphate-buffered saline, cells were homogenised in a small volume of TET-buffer (Tris 15 mM,

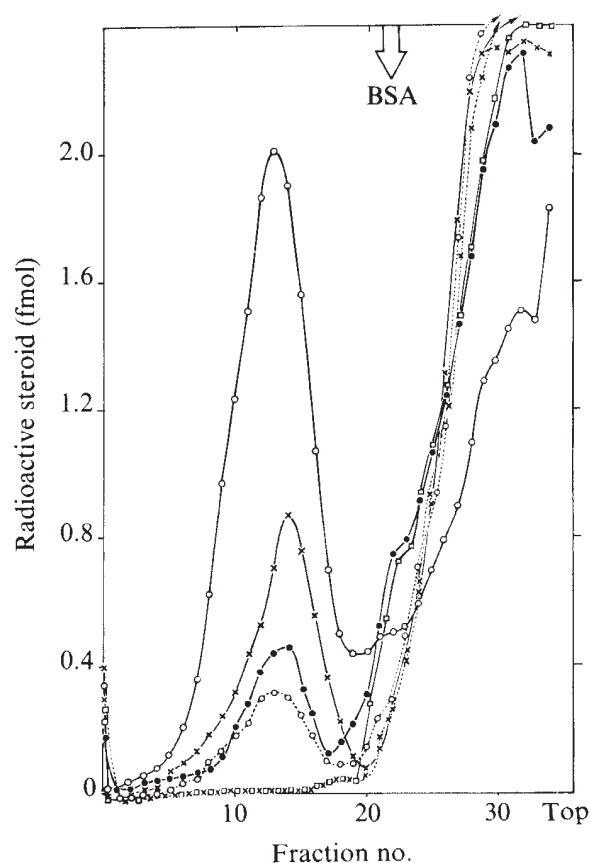


Fig. 1 Ultracentrifugation of L-929 cell cytosol preincubated with radioactive dexamethasone, androstanoalone and oestradiol on linear 5-35% glycerol density gradients in TET buffer. Cytosols were labelled at 0-2 °C for 3 h and 0.3 ml samples were centrifuged at 149,000g_{av} for 18 h at 0-2 °C. Thirty-four fractions (2 drops) were collected (abscissa), counted and the results expressed in fmol of radioactive steroid (ordinate). ○—○, 1 nM ³H-dexamethasone plus 100 nM unlabelled dexamethasone; ×—×, 1 nM ³H-androstanoalone; ×—×, 1 nM ³H-androstanoalone plus 100 nM unlabelled androstanoalone; ●—●, 1 nM ³H-oestradiol; □—□, 1 nM ³H-oestradiol plus 100 nM unlabelled oestradiol.

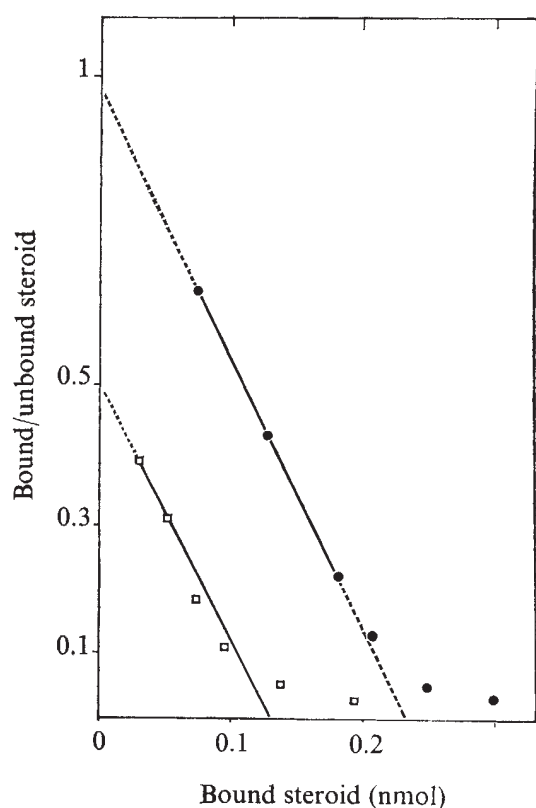


Fig. 2 Scatchard plot of binding data for androstanolone and oestradiol by L-929 cell cytosol. Binding of ^3H -androstanolone ($\bullet$) and ^3H -oestradiol ($\square$) in the 105,000g cytosol (4.9 mg protein per ml) from L cells, using increasing concentrations of steroid from 0.2 nM to 100 nM. Specific binding was determined after exposure to charcoal-dextran (5.0–0.5 mg ml $^{-1}$ TET buffer) at 0 °C for 30 min followed by centrifugation and counting of the supernatant.

EDTA 1 mM, dithiothreitol 1 mM, pH 7.4) in a glass-glass homogeniser. The cytosol fraction was obtained after alternate centrifugations at 700g for 15 min and 105,000g for 60 min. Looking for the cytosol receptor, we used purified ^3H -steroids: androstanolone (5 α -androstane-17 β -ol-3-one; 175 Ci mmol $^{-1}$), dexamethasone (9 α -fluoro-16 α -methyl-11 β , 17,21-trihydroxypregn-1,4-diene-3,20-dione; 27 Ci mmol $^{-1}$), 17 β -oestradiol (60 Ci mmol $^{-1}$), progesterone (48 Ci mmol $^{-1}$) and R5020 (17,21-dimethyl-19-nor-4,9-pregnandiene-3, 20-dione; 51.4 Ci mmol $^{-1}$). Binding was achieved after incubation at 0–2 °C for at least 2 h, mostly at 1 nM. Unlabelled compounds were used in isotopic dilution experiments to assess nonspecific binding by a subtraction method or to measure binding inhibition, and to calculate

the number of binding sites and the k_{D}^{eq} for a series of measurements made with different concentrations of steroids. Binding was evaluated either by using a charcoal suspension to remove free and rapidly released steroids, or by ultracentrifugation through linear 5–35% glycerol Tris gradients as described before^{4–6}.

A symmetrical radioactive 7–8S peak was obtained after labelling with 1 nM ^3H -androstanolone, ^3H -oestradiol or ^3H -dexamethasone. No binding was observed after incubation with progesterone or R5020, a progestational agent of high affinity for progesterone receptor in the uterus and mammary gland. As Fig. 1 shows, the radioactive 7–8S peak of R-G was greatest, while that of R-A was twice as high as the R-E peak. Addition of the corresponding unlabelled steroid (100 nM) to the 1 nM ^3H -tracer almost abolished the radioactive 7–8S peaks of R-A and R-E, while 15% of R-G binding remained in the 7–8S area. In the 4S region of the gradient, no binding was observed with ^3H -androstanolone but some nonspecific binding (not depressible by isotopic dilution) was seen with ^3H -oestradiol and ^3H -dexamethasone. Treatment with Pronase (1 mg ml $^{-1}$) for 2 h at 0 °C abolished the 7–8S peak of 1 nM ^3H -androstanolone and ^3H -oestradiol in the cytosol.

After incubation with various concentrations of steroid (0.25–100 nM), the Scatchard plot of the data (Fig. 2) indicated $k_{\text{D}} \sim 0.25$ nM for androstanolone and 0.20 nM for oestradiol with 47.8 and 23.5 fmol mg $^{-1}$ protein of cytosol (measured by the Lowry method), respectively, corresponding to about 3,000 and 1,500 sites per cell (calculated from the cell number).

Specificity studies by competition assays with unlabelled steroids (100 nM, Table 1) suggested that the three binding sites are probably associated with three distinct and non-interacting sites, and that they are similar to the oestrogen, androgen and glucocorticosteroid receptors described in many mammalian target organs^{4–6}. Oestrogens such as the non-steroid diethylstilboestrol (DES) compete with ^3H -oestradiol for receptors, but cortisol, progesterone and androgens do not interfere significantly with the binding of oestradiol. ^3H -androstanolone binding is inhibited by androstanolone and testosterone, and to a smaller extent by progesterone and cyproterone acetate, an anti-androgen (of the progesterone series), and by oestradiol, but neither DES nor cortisol competes significantly. These results cannot be explained by the presence of a single binding site, but they are compatible with two sites, R-A and R-E. The same strict oestrogen specificity for R-E has been observed in all oestrogen sensitive tissues studied so far, while competition by androgens, progesterone, anti-androgens and oestradiol, but not DES, has also been observed with all androgen receptors^{3,7–10}.

Furthermore, the small degree of competition between ^3H -dexamethasone and androstanolone or oestradiol, and conversely the virtual absence of competition on R-A and

Table 1 Specificity studies for steroid receptors in L-929 cells

Radioactive hormone (1 nM)	R-A ^3H -androstanolone	R-E ^3H -oestradiol	R-G ^3H -dexamethasone
No competitor*	100	100	100
Non-radioactive competitor (100 nM)			
Androstanolone	13	91	90
Testosterone	16	99	—
Cyproterone acetate	29	98	80
Oestradiol	47	16	90
DES	95	20	93
Progesterone	39	97	33
Cortisol	92	92	34
Dexamethasone	—	—	22

Samples of cytosol were incubated at 0–2 °C with ^3H -steroids with or without unlabelled steroids for 3 h, and bound radioactivity was obtained after 30 min of exposure to charcoal-dextran (as described in Fig. 2).

*100 expresses the binding with 1 nM ^3H -steroid and corresponds to 18.0 fmol bound androstanolone, 9.4 fmol bound oestradiol and 40.0 fmol bound dexamethasone per mg cytosol protein, respectively, for R-A, R-E and R-G.

R-E binding by cortisol favour independent R-G binding sites. No progesterone receptor was detected.

To investigate nuclear receptors, we obtained nuclei from the pellet of the homogenate after centrifugation at 700g for 15 min. Nuclei were suspended in 0.25 M sucrose, 1 mM MgCl₂ and 10 mM Tris buffer, pH 7.5, containing 1% Triton and washed three times in the same buffer without Triton. We looked for the nuclear receptor by incubating the nuclear suspension directly, or after labelling the 0.5 M KCl nuclear extract. In the first case, nuclei were resuspended in sucrose buffer and samples were incubated with 1 nM or 5 nM tritiated steroids, alone or with a 100-fold excess of the corresponding unlabelled compound for 1 h at 0 °C followed by 2 h at 20 °C. After washing three times with excess buffer followed by methanol extraction of the pellet, no specific binding was observed. By the other approach, the washed nuclear pellet was extracted with high salt buffer (TET-0.5 M KCl), centrifuged at 105,000g for 60 min and the soluble extract was incubated with 1 nM or 5 nM tritiated hormones and tested for binding by charcoal-dextran or density gradient centrifugations. No specific binding could be observed as in target tissue nuclei from hormone-deprived animals⁵⁻⁶. This was compatible with the presence of a very small or insignificant amount of steroid in the medium (verified by direct radioimmunoassays), and facilitated further study of hormonal effects on receptor distribution.

Cells were incubated for 30 or 60 min with 5 nM ³H-androstanolone or ³H-oestradiol at 37 °C in 5% CO₂ in air. A 5.3S peak was obtained in the 0.5 M KCl extract after gradient ultracentrifugation analysis, corresponding in the case of ³H-androstanolone to 20.6 fmol mg⁻¹ protein after 30 min, and 23.7 fmol mg⁻¹ after 60 min of incubation. In the cytosols, analysed on glycerol-Tris gradients, 4.6 fmol mg⁻¹ protein remained in the 7-8S peak after 30 min and 3.6 fmol mg⁻¹ after 60 min. After 60 min of incubation with ³H-oestradiol, the 5.3S nuclear peak corresponded to a protein concentration of 9.4 fmol mg⁻¹ and the 7-8S cytosol peak to 1.2 fmol mg⁻¹. As in other systems¹¹, about 40% of nuclear radioactivity was not extracted by KCl buffer, and was not studied further. When the cells were incubated together in 5 nM ³H tracer and 500 nM unlabelled steroid, no 5.3S peak was observed in the nuclear extract.

These results suggested that, as in all steroid-sensitive cells the cytosol receptors in L-929 cells are transferred into the nuclei in the form of ³H-steroid-receptor complexes after brief exposure of L cells to the appropriate hormone at 37 °C.

As the three cytosol receptors had the same S value on gradient ultracentrifugation, we investigated whether two or three of the different binding sites could belong to the same macromolecule. We reasoned that, if this is the case, the transfer of the oestradiol receptor into the nucleus would leave available androgen binding sites in the soluble nuclear extract (and vice versa for the transfer of R-A). But no binding was observed, after exposure of cells to 5 nM unlabelled oestradiol at 37 °C and secondary labelling of the nuclear 0.5 M KCl extract for 2 h at 0 °C with 1 nM ³H-androstanolone. Similar results were obtained with incubation in other steroids followed by hetero-labelling and all results were negative.

Therefore, we conclude that R-A, R-E and R-G are distinct binding molecules, as already observed for R-A and R-E in MI₁ cells³ and in the uterus and prostate^{12,13}. These data for cell lines such as MI₁ or L suggest that different receptors are present simultaneously in a single cell, an impossible conclusion for organs made up of various cell types. In the case of the uterus, however, the induction of oestradiol and progesterone receptors by progesterone and oestradiol, respectively¹⁴⁻¹⁶, favours the likelihood of a cellular multiplicity of types of receptor within a cell.

Thus by all criteria of binding affinity and specificity, physicochemical properties, cell distribution and nuclear transfer L-929 cells contain three typical steroid receptors, very similar to functional target organ receptors detected already. This supports the concept of cellular plurality of receptors, which in turn should be studied in terms of potentially different interactions with the genome^{6,17,18}. It is interesting that oestradiol, the native oestrogen, can, in a given cell, interact with two specific proteins, R-E and R-A, of different affinities, and that a synthetic oestrogen, DES of great therapeutic interest¹⁹, binds strongly to one of them, R-E, and very little to the other, R-A.

Are these receptors vestigial and unable to mediate steroid effects? Our preliminary observations of L-929 cells growing in the presence of steroids show that with 30 nM androstanolone, there is a moderate increase of growth rate. Moreover, after 10 d of suspension culture with this androgen, the amount of cytosol R-E per mg of cytosol protein decreases approximately 50% (which is not explained by an increase of nuclear binding sites). Conversely, after 10 d in 30 nM DES (17 β -oestradiol cannot be used because it binds to R-A), there is no decrease in R-A sites in the cytosol. Macromolecular syntheses (total proteins and DNA) are also changed by different steroid concentrations and combinations. Normal fibroblasts (ref. 20 and A. Groyer and P. Robel, unpublished) contain sex steroid receptor(s), and it will be interesting to see if these cells are more endocrinologically competent than previously believed.

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Hereditary persistence of foetal haemoglobin with β -chain synthesis in *cis* position (G γ - β^+ -HPFH) in a negro family

HEREDITARY persistence of foetal haemoglobin (HPFH) is an uncommon condition described mainly in negroes and Greeks. Although this genetic disorder is rare, it has been very important in the study of the arrangement of human globin genes on chromosomes and in the investigation of the regulation of foetal haemoglobin synthesis. It is not

always possible to distinguish clearly between HPFH and thalassaemia and there is considerable genetic heterogeneity in these conditions. A majority of patients with HPFH have an increased amount of foetal haemoglobin, normal or near normal red cell indices, Hb F in each red cell, an absence of clinical manifestations in the simple heterozygous state or in combination with Hb S, Hb C, or Hb E, and balanced globin synthesis¹. Two different forms of γ chains are normally synthesised, with either glycine or alanine at the γ^{136} position. Although the majority of negro heterozygotes for HPFH have both glycine and alanine at γ^{136} , several patients have had only glycine²⁻⁵. There was no synthesis of β or δ chains in the *cis* position to the HPFH gene in any of the appropriate negro cases reported previously. We describe here a negro family in which the proposita had Hb S-HPFH with β -chain synthesis in the *cis* position to the HPFH gene.

In a screening study conducted in high schools in Philadelphia, an asymptomatic female teacher with unusual proportions of Hb S, Hb A, and Hb F was detected by cellulose acetate electrophoresis. The Hb S level was 40.9%, Hb F 29.7%, Hb A 27.5% and Hb A₂ 1.9%. The presence of Hb S was confirmed by the dithionite-phosphate solubility test⁶ and by precipitation during shaking of the oxy-form of the haemoglobin⁷. On acid elution of peripheral blood smears⁸ all her red cells were found to contain Hb F, but in varying amounts. Further study of the patient revealed that she had never experienced symptoms of sickling, but that her red cell morphology as well as her red cell indices and reticulocyte count were normal. A study of her family (Table 1) showed that her husband, her putative father and her sister were normal, but that her 9-month-old son had an Hb F level of 22.1% and an Hb A₂ of 1.1% with normal red cell indices and Hb F present in every red cell. Her mother had sickle cell trait with Hb S of 40.0%. A study of 12 different blood group systems in the proposita and her parents failed to exclude paternity. Globin synthesis studies were performed on each member of the family (Table 1). The results showed balanced globin synthesis in each individual, a result in agreement with the normal red cell indices in failing to detect evidence of thalassaemia in this family. In the proposita the β^A/α and β^S/α specific activity ratios were near unity (1.06, 1.08), results different from the decrease in one or both of these ratios found in negroes with Hb S- β^+ thalassaemia¹⁰. Analysis of the glycine and alanine residues in the third portion of the γ chain after cyanogen bromide cleavage¹¹ gave values in the proposita of glycine 1.02 and alanine 1.97, and in her son of glycine 1.07 and alanine 2.01. These findings may be interpreted as consistent with production of chains with only glycine at γ^{136} .

The presence of HPFH in the proposita and her son is indicated by Hb F being present in all red cells, normal haematological values, balanced globin synthesis and the lack of interaction with Hb S. This family is of special interest in that β -chain production is present and directed

by the chromosomes which contains the HPFH determinant, in contrast to all other negro varieties previously described. The HPFH gene in the negro family described here differs from the British¹² and Greek¹³ types in the higher amount of Hb F produced and in the content of only glycine at γ^{136} . Production of β^A chain by the affected chromosome probably occurs in all three varieties. Although the child of the proposita clearly has HPFH, neither parent of the proposita has this condition. Non-paternity was not demonstrated by the studies performed, but either this or a mutation are possibilities in this family.

The majority of patients with HPFH have balanced globin synthesis, although three negro heterozygotes and one homozygote have had clearly decreased ratios^{1,12,14,15}. Although many negro heterozygotes with β thalassaemia have normal globin synthesis ratios^{16,17}, the findings in this family are not consistent with the presence of β thalassaemia; nor are the normal globin synthesis studies and haematological values¹⁸.

Studies involving patients with haemoglobin Kenya have indicated that the genes for γ , δ and β chains are closely linked on the same chromosome^{19,20}. In the previously described forms of negro HPFH the findings may be attributed to the deletion of the δ and β chain loci, together with some controlling regions which allow continuing synthesis of $^G\gamma$ and $^A\gamma$, or only $^G\gamma$. In the present family, the low Hb A₂ values suggest that the δ -chain locus may be deleted. The total deletion in this family may therefore involve the $^A\gamma$ and δ loci and some controlling regions, leaving $^G\gamma$ and β chain synthesis intact. It is of interest that in the proposita the percentage of Hb S is almost identical to that found in her mother and similar to that usually found in sickle cell trait, demonstrating that the combined synthesis of Hb F and Hb A directed by the HPFH chromosome is similar to the synthesis of Hb A seen in sickle cell trait and greater than the synthesis of Hb S. As observed in this patient and in other negro heterozygotes for Hb A-HPFH or Hb S-HPFH, the limit of Hb F synthesis per cell seems to be approximately 30–35%. In patients with Hb S- β^0 -HPFH, the normal haemoglobin content of the cell is achieved by compensation from the β^S gene, which is responsible for approximately 70% of the total haemoglobin production. In Hb S- β^+ -HPFH described here, Hb F still comprises only 30% of the total haemoglobin in the cell, although Hb A is now responsible for the compensatory synthesis which results in a normal mean cell haemoglobin value. Another family with similar characteristics has recently been found²¹.

The presence of Hb A in the proposita indicates that genetically directed Hb synthesis may persist in each red cell without loss of the β chain cell locus. The importance of this finding is that a variety of defects seem to control HPFH, suggesting that a search for the biochemical defects in these various conditions may provide clues for influencing the synthesis of Hb F in persons who retain β chain

Table 1 Haematological and globin synthesis data on family members

Subject	Age	Hb (g dl ⁻¹)	MCV (μ m ³)	MCH (pg)	Phenotype (Hb)	Hb A ₂ (%)	Hb F (%)	non- α/α
Proposita	27	12.0	92.0	30.6	SFA	1.9	29.7	1.08
Husband	28	15.2	92.0	31.5	A	2.9	0.1	0.92
Son	9/12	11.7	82.0	27.7	AF	1.1	22.1	1.17
Sister	23	12.3	90.0	29.4	A	2.4	0.4	1.06
Mother	56	13.1	88.0	29.7	AS	—	0.3	1.03
Father	67	14.4	88.0	29.7	A	2.9	0.8	0.97

The non- α/α ratio was determined after incubation of peripheral blood with ¹⁴C-leucine for 2 h at 37 °C, preparation of haemolysate and separation of the globin chains by chromatography on carboxymethyl cellulose in 8 M urea at pH 6.5 with a sodium phosphate gradient⁹. The total radioactivity in α , β S and β A globin peaks was summed when these chains were present in the sample, and was divided by the α -globin peak to obtain the non- α/α ratio.

loci, such as those with sickle cell disease and thalassaemia major.

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Permeability of the blood–cerebrospinal fluid barrier to plasma proteins during foetal and perinatal life

HIGH levels of alpha foetoprotein (AFP), albumin and IgG have been detected in the cerebrospinal fluid (CSF) of six human foetuses between 16.5 and 25 weeks old¹. The levels of AFP were found to decline from 1,200 $\mu\text{g ml}^{-1}$ in a foetus of 16.5 weeks to 52 and 60 $\mu\text{g ml}^{-1}$ in the older ones tested. During this period of foetal life, the ratios between albumin and AFP in CSF were shown to increase. The levels of IgG ranged between 60 and 117 $\mu\text{g ml}^{-1}$ and were higher than those detected in CSF from normal adult subjects (20–40 $\mu\text{g ml}^{-1}$)².

These preliminary results have prompted us to extend our investigations to CSF from a larger group of human foetuses and compare the levels of albumin, AFP and IgG with those of total protein, transferrin, β -trace protein^{3,4}, β 2-microglobulin⁵ and lysozyme⁶. Samples of CSF were obtained from 13 foetuses between 14 and 25 weeks old and one sample from a foetus 40 weeks old with hydrocephaly and severe achondroplasia (Table 1). Some of these foetuses were obtained by hysterotomy and had chromosome abnormalities; others were aborted spontaneously and apparently normal. One was a male foetus obtained by therapeutic abortion from a woman carrier of a gene for haemophilia.

CSF was collected by aspiration with a syringe from the cisterna magna; only CSF samples which were absolutely clear from contamination by blood were tested.

The levels of albumin, transferrin, IgG and β -trace protein were estimated by the single radial diffusion technique⁷; AFP was measured by the one dimensional antigen–antibody electrophoresis (rocket technique)¹; lysozyme by

the lysoplate method⁸ and the levels of β 2-microglobulin by radioimmunoassay (Phadebas, β 2-microtest, Pharmacia).

The levels of AFP were higher in CSF of foetuses less than 20 weeks old than in the older foetuses; in two foetuses 16.5 and 19 weeks old the levels were more than 1 mg ml^{-1} , whereas in three foetuses 25 weeks old the values of AFP varied between 17 and 52 $\mu\text{g ml}^{-1}$ (Table 1). In the 40-week-old foetus, the concentration of AFP in CSF was less than 1 $\mu\text{g ml}^{-1}$. The levels of albumin and the ratios of albumin to AFP were higher in foetuses between 20 and 25 weeks old than in the younger foetuses (Table 2). Ratios between 1.8 and 4.7 were observed when the levels of total proteins and those of albumin were compared.

IgG was detected in all foetuses studied and slightly higher levels were observed in samples from foetuses between 20 and 25 weeks old. β 2-Microglobulin was found in all samples tested (Table 1) and the ratio IgG/ β 2-microglobulin showed only minor variations, ranging between 48 and 116 (Table 2). Lysozyme could not be detected in the foetal CSF using the lysoplate technique; β -trace protein, was found in all foetal CSF and appeared to be a major protein of this body fluid.

In summary, all the proteins tested, with the exception of lysozyme, have been found in foetal CSF in concentrations much higher than those detected in CSF from normal adult subjects. These results, therefore, support the suggestion that the human blood–CSF barrier is not fully developed during foetal life¹ and explain the detection of the high levels of IgG and the presence of specific maternal antibodies in CSF of human infants tested during the first weeks of life^{8–10}.

The presence of high levels of AFP in foetal CSF also supports the view that the increased amount of this foetal protein detected in the amniotic fluids of foetuses with open neural-tube defects is due to the transfer of AFP from CSF into the amniotic cavity^{1,11,12}.

In view of the high levels of proteins detected in CSF from human foetuses, the transfer of labelled proteins from the blood to the CSF was investigated in newborn and adult rats (M.A. and S.H.). Albumin and IgG were isolated from adult rat serum by precipitation with ammonium sulphate, DEAE chromatography and gel filtration on Sephadex G-200 (ref. 7). Rat IgG antibodies against brain antigens were isolated, using similar techniques, from an immune serum given by Dr R. Hughes, Guy's Hospital Medical School. ¹⁴C-Oestrogen was purchased from the Radiochemical Centre (4-¹⁴C-oestradiol, 10 μCi per 510 ml).

Rat albumin, and rat IgG from normal rat serum and immune serum were labelled with ¹²⁵I (specific activity 14 mCi mg^{-1} iodine, Amersham) using the chloramine T method¹³. The extent of ¹²⁵I incorporation was assayed after precipitation of the labelled proteins—in the presence of carrier protein—with 10% trichloroacetic acid (TCA) and by the analysis of radioactivity in strips obtained from cellulose acetate electrophoretic patterns, as previously described¹⁴.

The labelled proteins were injected into the peritoneal cavity of newborn rats, on the first, third, fourth and seventh day after birth. The newborn animals—and the adult rats, used as controls—were killed between 3 and 24 h after the injections and blood was collected from the jugular vein. Samples of CSF, free of blood, were collected from the cisterna magna¹⁵.

In the 1-d-old rats about 20 and 10% of ¹²⁵I-albumin and ¹²⁵I-IgG, respectively present in blood, were detected in CSF 3 h after the injections into the peritoneal cavity. The labelled proteins could not be detected in CSF obtained from rats 7 d old or from adult rats, 3 h after the injections.

Figure 1 shows the results of one of the experiments in which ¹²⁵I-IgG isolated from normal rat serum or from rat immune serum containing antibodies against rat brain antigens, were used. Two newborn rats were injected intraperitoneally with ¹²⁵I-IgG and two newborns with ¹²⁵I-IgG

Table 1 Levels of plasma proteins in foetal CSF

Foetal age (weeks)	Total proteins (mg ml ⁻¹)	Albumin (µg ml ⁻¹)	AFP (µg ml ⁻¹)	Transferrin (µg ml ⁻¹)	IgG (µg ml ⁻¹)	β2-Microglobulin (µg ml ⁻¹)	Causes of abortion
14	1.10	380	650	NT	25	NT	S
14	1.25	400	720	NT	NT	NT	S
16.5*	NT	NT	1,220	NT	NT	NT	S
18*	2.05	1,024	420	10	72	1.02	T
19	2.75	1,050	600	35	63	0.96	S
19.5*	NT	NT	1,040	NT	NT	NT	S
18-20	4.50	1,930	600	40	60	1.24	T
20	7.70	4,100	625	150	168	1.68	S
23.5	5.90	3,000	190	80	163	1.40	T
24	7.30	3,030	160	140	109	NT	T
25*	3.02	1,200	60	25	109	1.56	T
25*	3.95	840	71	NT	117	1.09	T
25*	3.20	680	52	10	60	0.66	T
40	2.5	NT	1	NT	71	NT	S
Normal adult CSF†	—	140-200	—	10-30	20-40	—	—

NT, not tested; S, spontaneous; T, therapeutic.

* Levels of AFP already reported¹.† From Laterre (ref. 2); mean levels (µg ml⁻¹).

rat anti-rat brain. After 3 h the animals were killed and the radioactivity was measured in blood and CSF. Another four newborn rats from the same litter were injected when 4 d old and the remaining three at 7 d of age. About 10% of ¹²⁵I-IgG present in blood was detected in the CSF from 1-d-old rats. In the newborn rats injected with rat IgG antibodies against brain antigens the CSF contained over 20% of the labelled protein present in blood. Higher values of labelled IgG were detected in the CSF of rats 4 d old injected with rat antibodies than in rats injected with normal IgG. In rats 7 d old, less than 1% of the labelled protein present in blood was detected in the CSF of the animals killed 3 h after injection (Fig. 1).

More than 96% of the radioactivity present in serum and CSF was precipitated by TCA. When these samples were analysed by cellulose acetate electrophoresis, the peaks of radioactivity in serum and CSF were detected in the area corresponding to the IgG region in the electrophoretic patterns.

Preliminary studies have also indicated that an equilibrium between the levels of radioactive albumin or IgG in blood and CSF is reached within 24 h of the intraperitoneal injection into 1-d-old rats.

¹⁴C-Oestradiol, injected intraperitoneally in newborn rats, was detected in CSF 4 h after injection; the amount of labelled hormone found in CSF was between 40 and 60% of that detected in blood.

These studies and the results of preliminary investigations using heterologous antibodies against rat brain antigens (M.A. and S.H.) have thus confirmed that the permeability of the blood-CSF barrier is not fully developed during foetal and perinatal life in man and rats^{1,16}.

Table 2 Ratios of levels of proteins in CSF

Foetus	Total protein/albumin	Alb./AFP	Alb./IgG	IgG/β2-microglobulin
1	2.89	0.58	15.2	—
2	3.12	0.55	—	—
3	—	—	—	—
4	2.00	2.43	14.22	70
5	2.61	1.75	16.66	65
6	—	—	—	—
7	2.33	3.21	32.16	48
8	1.87	6.65	24.40	100
9	1.96	15.78	18.40	116
10	2.40	18.13	27.79	—
11	2.51	20.00	11.09	69
12	4.70	11.83	7.11	107
13	4.70	13.07	11.33	90
14	—	—	—	—

It has long been recognised that the development of brain and unfolding of behaviour are sensitive to modifications by environmental factors¹⁷.

Experimental studies, reviewed by Balazs¹⁸, have shown that abnormal hormonal states during foetal life can result in permanent behavioural disorders, whereas, in adults, they usually induce changes that are reversible.

We suggest that, in abnormal maternal states, the permeability of the foetal blood-CSF barrier to hormones and antibodies against antigens of the nervous system may have an important role in the production of long lasting damage to the developing brain and to the unfolding of behaviour. Experimental work in progress should confirm or disprove this hypothesis.

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Antibodies to native tRNA in NZB/NZW mice

ANTIBODIES to native DNA, double-stranded RNA, and various synthetic polyribonucleotides occur with great frequency in patients with systemic lupus erythematosus (SLE)¹. These antibodies are also present in the New Zealand mouse, particularly the NZB/NZW F₁ hybrid,

which is considered a good animal model for human SLE². We report here the presence and characterisation of antibodies that bind tRNA, specifically in its native conformation, in sera from old NZB/NZW mice.

Figure 1 shows the results of gel chromatography experiments that demonstrate the presence of anti-tRNA antibodies in old NZB/NZW female mouse serum. The serum was treated to eliminate ribonuclease activity, incubated with radioactive *E. coli* tRNA, and then chromatographed on a small column of Sephadex G-100. Protein-bound tRNA was excluded from the gel while unbound tRNA was retarded (Fig. 1b). Control mice serum showed virtually no binding (Fig. 1a). We could also demonstrate tRNA binding activity in the NZB/NZW sera, but not in controls, using ammonium sulphate precipitation³ or by precipitation of the complex with a rabbit anti-mouse antibody preparation.

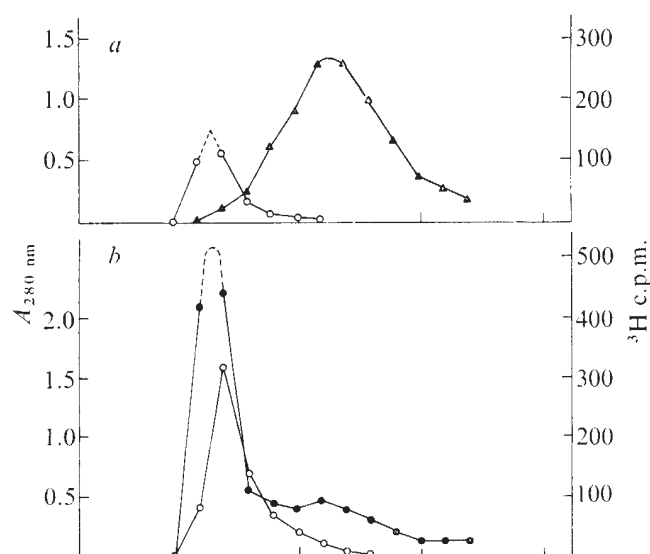


Fig. 1 NZB/NZW F₁ hybrids and BALB/c control strains were obtained from colonies maintained at the National Institutes of Health. Mice were bled by orbital sinus puncture. About 20 sera were pooled, heated to 56 °C for 30 min and the immunoglobulin fraction was precipitated by (NH₄)₂SO₄ at 50% saturation. This fraction was treated with charcoal to remove ribonuclease activity (manuscript in preparation) and then dissolved in phosphate-buffered saline to the volume of the original serum. Reaction mixtures contained 0.1 ml of serum, 0.15 µg (2,000 c.p.m.) of *E. coli* ³H-tRNA (obtained by growing bacteria in medium containing ³H-uridine), and 0.01 M MgCl₂ in a final volume of 0.12 ml. The mixture was incubated for 1 h at 4 °C and then applied to a 0.7 × 30 cm Sephadex G-100 column. Chromatography was at 4 °C. Fractions of 0.65 ml were collected, measured for absorbance at 280 nm and counted in a scintillation counter. *a*, Incubation of ³H-tRNA with normal serum; *b*, incubation of ³H-tRNA with 8-month-old NZB/NZW female mouse serum. ○, A₂₈₀; ●, ³H-radioactivity.

Figure 2 shows the capability of the serum to bind essentially all added tRNA, indicating that the antibody recognises a general feature of all native tRNAs and not one or more of the rare nucleosides. (Alternatively, there may be subpopulations of antibodies which recognise each amino acid-specific tRNA.) Studies of antibodies made to conjugated yeast phenylalanine tRNA showed that they recognised only a modified nucleoside in the tRNA⁴. Figure 3 shows the age dependence of tRNA binding activity in the sera of female NZB/NZW mice. Each point represents the binding activity of sera pooled from 20 mice. The abrupt increase in binding at between 5 and 7 months suggests that these hybrid mice are programmed genetically

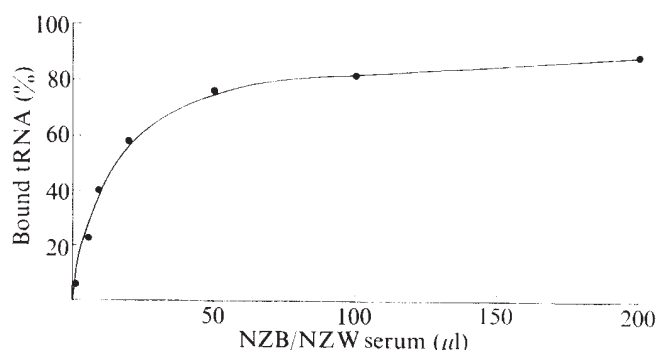


Fig. 2 Binding curve of ³H-tRNA to 8-month-old NZB/NZW mouse female serum. Increasing amounts of serum were incubated with a fixed amount (75 ng) of ³H-tRNA. Binding capacity was measured by Sephadex chromatography as described in the legend to Fig. 1.

to produce anti-nucleic acid antibodies at a particular age. The production of anti-tRNA antibodies is very well correlated with other manifestations of the sickness², such as renal immunoglobulin deposition, renal histological abnormalities, proteinuria and the production of anti-DNA antibodies⁵. The decline in tRNA binding activity after 10 months of age may result from the death of the most severely ill animals, decreased production or increased catabolism of the antibodies⁶.

NZB/NZW female mice (8–9 months old) sera were also assayed individually for tRNA and DNA-binding activity. All sera that bound DNA were also positive for tRNA. Five out of seven selected on the basis of very low double-stranded DNA binding (indistinguishable from normal mice), however, showed substantial binding for tRNA as well as for single-stranded DNA. These results suggest that anti-tRNA antibodies are more characteristic of old NZB/NZW mice than anti-native DNA antibodies.

Since the tRNA-binding protein in old NZB/NZW mouse serum is produced spontaneously in pathological conditions and not in response to a given immunogen, we tried to demonstrate that this protein is an immunoglobulin

Table 1 Inhibition of ³H-tRNA and ¹⁴C-DNA-binding to NZB/NZW sera by nucleic acids and nucleic acid components

Inhibitor	Inhibition of ³ H-tRNA-antibody complex (%)	Inhibition of ¹⁴ C-DNA-antibody complex (%)
<i>E. coli</i> tRNA	63	25
Rat liver tRNA	63	
Denatured <i>E. coli</i> tRNA	25	34
Uridine	0	5
Cytidine	0	18
5'-Adenosine monophosphate	0	15
poly(A)	0	16
poly(rA)-poly(rU)	0	7
poly(rI)-poly(rC)	5	30
Double-stranded DNA	15	93
Single-stranded DNA	0	100
MS2 viral RNA	38	22
Qβ-viral RNA	38	8
Denatured MS2 viral RNA	13	20
16S ribosomal RNA	41	35
5S ribosomal RNA	43	26

³H-tRNA (0.15 µg) or native KB cell ¹⁴C-DNA⁵ (0.05 µg) were mixed with 1.5 µg of each of the specified inhibitors and NZB/NZW serum (100 µl serum for the ³H-tRNA assay and 10 µl serum for the ¹⁴C-DNA assay). Incubation was for 60 min at 4 °C. tRNA binding was analysed by Sephadex chromatography, as described in the legend to Fig. 1. DNA binding was measured by ammonium sulphate precipitation at 35% saturation. Denatured tRNA and denatured MS2 RNA were prepared by the method of Axelrod *et al.*¹³. MS2 RNA was given by Dr Rushizky, National Institutes of Health.

and not another binding protein or an enzyme. We used a multivalent rabbit anti-mouse γ globulin which had been purified by affinity chromatography (given by Dr R. Asofsky). Addition of 0.6 mg of this immunoglobulin to 50 μ l of the NZB/NZW serum, incubation at 4 °C for 5 d and centrifugation decreased the subsequent binding activity for 3 H-tRNA by 96%. Furthermore, the purified anti-mouse γ globulin could precipitate all the tRNA bound to NZB/NZW serum in 2 h at 4 °C.

The specificity of old NZB/NZW mouse serum with respect to different nucleic acid antigens was tested by competition experiments (Table 1). The same antigens were also used to inhibit complex formation between anti-DNA antibodies and native 14 C-DNA. The antibodies in NZB/NZW serum that bound tRNA and DNA behaved as distinct antibody populations, as the inhibition patterns of the two populations by different nucleic acids were very different (Table 1). 3 H-tRNA binding was best inhibited by tRNA while 14 C-DNA binding was best inhibited by double-stranded and single-stranded DNA.

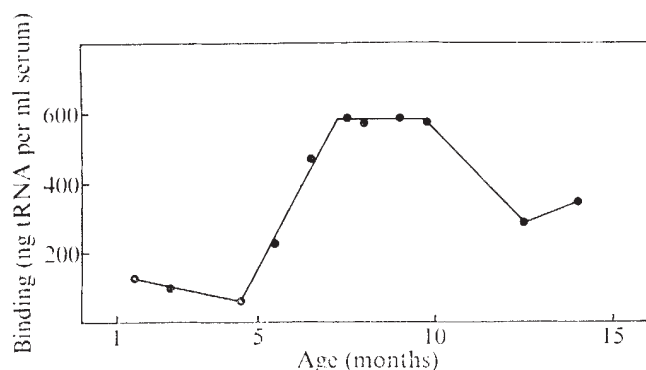


Fig. 3 About 20 female NZB/NZW mice of each age were bled and the sera were pooled, and assayed for tRNA-binding activity by Sephadex chromatography as described in Fig. 1.

Rat liver tRNA inhibited the tRNA-antibody reaction to the same extent as did *E. coli* tRNA. This supports the contention that the antibodies recognise a general feature in the structure of tRNA and not minor components which are somewhat different in the two species.

On the other hand, denatured tRNA which was fixed in its denatured conformation by formaldehyde, did not effectively compete with native tRNA, so this serum shows a marked specificity for the native conformation of tRNA. Nucleosides and nucleotides did not inhibit the binding reaction at all. The double-stranded synthetic polynucleotide poly(rI)-poly(rC) and DNA, which both bind very well to sera of NZB/NZW mice and SLE patients⁶, inhibited tRNA-binding to a very small extent. Conversely, single-stranded viral RNAs and ribosomal RNAs, which are believed to form hairpin secondary structures similar to those of tRNAs^{7,8}, competed fairly effectively. In fact, 5S rRNA has been shown to compete with tRNA for a ribosomal binding site⁹ and some viral RNAs can be aminoacylated by aminoacyl tRNA synthetases¹⁰. Denatured MS2 RNA cross linked with formaldehyde was a poor inhibitor compared with native MS2 RNA.

Attempts have been made in many laboratories to elicit antibodies specific for tRNA in experimental animals^{11,12}. None of these antibodies has shown evidence of specificity for the secondary or tertiary conformation of the tRNA. Such antibodies would be useful for studies of the conformation and function of free and complexed tRNA in solution. The antibodies in the sera of the NZB/NZW mice may be useful in this connection and they should be

of value in understanding the molecular basis of lupus-like diseases.

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Genetic control of type C virus of wild mice

THE genetic control of type C virus group-specific (GS) antigen expression^{1,2}, infectious virus³⁻⁶, and tumorigenesis^{6,7} has been described in crosses between strains of inbred laboratory mice with high and low incidence of leukaemia. We described a natural population of wild mice (*Mus musculus*) near Lake Casitas (LC) in southern California which showed a high level of indigenous type C virus activity in terms of GS antigen and infectious virus throughout their lifetime. When ageing was observed in the laboratory, LC wild mice showed a marked susceptibility to lymphoma⁸, epithelial tumours⁹ and a neurogenic hind leg paralysis¹⁰. Virus transmission and *in vivo* neutralisation studies¹¹ established the indigenous type C virus as the critical determinant of the lymphoma and paralysis. To ultimately prevent these diseases by antiviral measures we have attempted to suppress LC virus expression by cross-breeding LC wild mice with a specific inbred laboratory mouse strain C57BL/10 Snell (designated B10). The B10 mouse was selected because of its homozygosity for two non-linked dominant alleles, *Fv-1^b* and *MLv-1^b*, the function of either of which might be expected to restrict virus expression in the hybrid progeny. Laboratory strains of mouse type C virus can be classified according to their *in vitro* host range as N-, B-, or X(xenotropic)-tropic depending on whether they grow preferentially in NIH Swiss or BALB/c embryo cells or replicate only in non-murine cells^{12,13}. Field isolates from LC mice have an unusually wide *in vitro* host range ('amphotropic') but are N-tropic for mouse cells (S. Rasheed, V.K., and M.B.G., unpublished). B10 mice, within the first year of life, show a low incidence of both N- and B-tropic infectious virus¹⁴ and also harbour an endogenous X-tropic genome¹⁵. The *Fv-1^b* allele from the B10 parent should limit the replication of N-tropic virus within the LC×B10 progeny¹⁵, presumably by interfering with the integration or transcription of the proviral DNA¹⁶. The *MLv-1^b* allele, the function of which is unknown, might restrict either N-, B-, or X-tropic virus expression in the progeny since this allele completely suppressed GS antigen expression in B10 crosses with the

congenic resistant (58N) strain¹⁷. Although the genetic mechanism has yet to be established, our findings indicate that control of type C virus expression can be accomplished by genetic means in wild mice.

Parental 6-month-old B10 mice (Jackson Laboratory) were reciprocally bred with newly trapped LC wild mice. Based on their weight of 10–15 g the LC parental mice were estimated to be 6 months old. A laboratory breeding colony of LC mice was also established. Parental mice were splenectomised before breeding and the segregating progeny from the various crosses (Table 1) were splenectomised at 4–6 weeks of age. Spleen extracts (10% w/v) were tested for GS (p30) antigen by the complement fixation (CF) test. Six male and five female LC mice whose spleens were positive for GS antigen were selected for the parental mating with separate B10s. All the parental and backcross B10 mice showed an absence of detectable spleen GS antigen.

The segregation of GS antigen expression in the different crosses is shown in Table 1. In striking contrast to the high prevalence and titre of detectable spleen CF GS antigen in the LC parental (72.6% GS positive, 49.5% $\geq 1:4$), and 4–6-week-old LC mice born in the laboratory (66.7% GS positive, 40.0% $\geq 1:4$), there was an almost complete absence of detectable spleen GS antigen in the 4–6-week-old reciprocal LC $\times$ B10 F₁ hybrids (1.4%) and in the reciprocal F₁ (2.3%) and F₂ (1.7%) B10 backcrosses. The negligible occurrence of detectable GS antigen at weanling age in the F₁ hybrids and B10 backcross progeny indicates a dominant negative effect on this form of viral expression.

To determine if suppression of GS antigen expression was temporary or long lasting, we tested 10% extracts for

Table 1 Type C virus GS antigen expression in crosses between LC wild mice and B10 inbred mice

Generation of cross†	Spleen GS antigen (CF)*			
	1:2	%	$\geq 1:4$	%
Parental strains:				
B10	0/73‡	—	0/73	—
LC	207/289	72.6	143/289	49.5
F ₁ generation:				
B10 $\times$ LC	1/111	—	0/111	—
LC $\times$ B10	1/37	1.4	0/37	—
F ₂ generation:				
(B10 $\times$ LC)F ₁ $\times$ (LC $\times$ B10)F ₁	4/110	3.7	1/110	—
(LC $\times$ B10)F ₁ $\times$ (B10 $\times$ LC)F ₁	7/106	6.6	1/106	—
(B10 $\times$ LC)F ₁ $\times$ (B10 $\times$ LC)F ₁	11/115	9.6	1/115	—
(LC $\times$ B10)F ₁ $\times$ (LC $\times$ B10)F ₁	27/153	17.6	11/153	—
F ₁ backcross to B10:				
B10 $\times$ (B10 $\times$ LC)F ₁	0/62	—	0/62	—
B10 $\times$ (LC $\times$ B10)F ₁	1/36	—	1/36	—
(B10 $\times$ LC)F ₁ $\times$ B10	1/67	2.3	0/67	—
(LC $\times$ B10)F ₁ $\times$ B10	3/56	—	2/56	—
F ₂ backcross to B10:				
B10 $\times$ (B10 $\times$ LC)F ₂	1/29	—	0/29	—
B10 $\times$ (LC $\times$ B10)F ₂	0/9	—	0/9	—
(B10 $\times$ LC)F ₂ $\times$ B10	—	1.7	—	—
(LC $\times$ B10)F ₂ $\times$ B10	0/22	—	0/22	—
F ₁ backcross to LC:				
LC $\times$ (B10 $\times$ LC)F ₁	12/33	36.4	3/33	—
LC $\times$ (LC $\times$ B10)F ₁	17/36	47.2	7/36	—
(B10 $\times$ LC)F ₁ $\times$ LC	4/91	4.4	1/91	—
(LC $\times$ B10)F ₁ $\times$ LC	17/51	33.3	10/51	—
F ₁ laboratory bred LC:				
LC $\times$ LC	50/75	66.7	30/75	40.0

* Spleens were removed from parental LC and B10 mice in young adulthood (2–6 months of age) and from progeny mice at 4–6 weeks of age. 10% extracts were tested by CF at 1:2 and 1:4 antigen dilutions with a 1:2 dilution of rat sera pools (MVS 30, 3490, 4918) prepared in Fischer rats bearing Moloney sarcoma virus tumour transplants. Specificity of sera confirmed by comparison with guinea pig antisera to electrofocus purified MuLV (P30) antigen²².

† Terminology used in these crosses designates female on left and male on right of $\times$.

‡ Number positive/number tested.

Table 2 Type C virus isolation from wild-inbred parental and hybrid mice

Generation or cross	Spleen CF GS positive		Spleen CF GS negative	
	Spleen	Tail	Spleen	Tail
B10 parental	—	—	1/10	1/5
LC parental	4/4	3/5	—	—
F ₁	1/2	0/2	0/10	1/10
F ₂	1/13	0/10	0/24	0/10
F ₁ backcross B10	1/1	0/5	0/9	0/5
F ₁ backcross LC	9/9	4/6	0/9	0/2

For virus isolation 0.1 ml 10% spleen extract (stored at -70°C for several months) and 0.1 ml fresh 20% tail homogenates (not necessarily from the same individual mice) were assayed undiluted for induction of CF GS antigen after 21 d (COMUL test) on wild mouse SC-1 (ref. 20) and rabbit SIRC²¹ cells. Antisera used were as in Table 1.

CF GS antigen from a random sample of healthy F₁ progeny and F₁ backcrosses to B10 progeny after various periods of observation. We have shown that in 3-week-old LC mice, the degree of type C virus expression in the liver is equal to that in the spleen (M.B.G., V.K. and R.J.H., unpublished). GS antigen was detected in none of 23 F₁ hybrids and 93 F₁ backcross to B10 progeny observed for an average of 7 months (range 3–10 months). The repression of GS antigen thus seemed fairly long lasting.

In the F₂ reciprocal crosses, spleen GS antigen was found in an average of 10.0% (range 3.7–17.6%), mostly in low titre (1:2), (Table 1). Although these results suggested a two gene suppressive effect on GS antigen expression which would have predicted 7% GS antigen positive in the F₂ generation, we could not confirm this hypothesis by analysis of the GS antigen ratios in the F₁ backcross to LC mice. A greater prevalence of spleen GS antigen was observed in backcross matings with LC females (average 42.0%) compared with LC males (average 14.8%), (Table 1). This finding suggested either a positive maternal effect on virus expression by epigenetic spread of virus to offspring from the heavily infected LC maternal reproductive tract (M.B.G., V.K. and R.J.H., unpublished) and milk¹⁸ or a negative maternal effect, presumably from neutralising antibodies passed to their offspring by the virus-antigen-stimulated hybrid mothers⁴. In the latter instance the only segregation data that would be directly interpretable are those backcross matings with LC mothers. Viewed in this way our results are most compatible with segregation of a single gene, that is, 42% observed compared with 50% predicted GS-positive progeny in the LC maternal backcross. The single gene probably responsible for this suppression of GS antigen would be the *Fv-1* rather than *MLv-1^b* since the *Fv-1* locus has been shown to be the major determinant of virus expression and spontaneous leukaemia in hybrids with AKR and other high-leukaemia *Fv-1^a* laboratory mouse strains^{4,6}. A more extensive genetic analysis will be required, however, to rigorously establish the gene locus primarily responsible for this dominant control of virus expression in LC wild mice.

A sample of the 10% spleen extracts collected at 4–6 weeks of age and previously tested for GS antigen by CF, and fresh 10% tail clip homogenates collected at 6–12 months of age were assayed for infectious virus by the COMUL test¹⁹ on mouse (SC-1) and rabbit (SIRC) cells (Table 2). SC-1 cells (Dr J. Hartley, NIAID, NIH) are a cloned wild mouse embryo cell line which seem to lack both the *Fv-1^a* and *Fv-1^b* alleles and are thus equally susceptible to N- and B-tropic viruses²⁰. SIRC cells are a rabbit cell line²¹ susceptible to the X-tropic viruses of laboratory mice and the amphotropic viruses from LC wild mice (S. Rasheed, V.K., and M.B.G., unpublished). Virus was recovered in both SC-1 and SIRC cells from all the high titred ($\geq 1:4$) GS-positive spleens (13 of 13) and from most (7 of 11) of the tail homogenates tested from the parental and backcross LC mice. By contrast,

virus was recovered in only SC-1 cells from just 6 of 116 total assays from spleen and tail of parental B10 and F₁, F₂, and B10 backcross progeny, including a sample from those few mice in these groups whose spleen extracts were weakly positive (1:2) for CF GS antigen. Thus, infectious amphotropic virus could readily be recovered from spleen or tail only of parental and backcross LC mice which had high titred spleen GS antigen. In no instance was virus isolated on SIRC and not on SC-1 cells. Therefore, it is unlikely that any of the other isolates represented the X-tropic class of endogenous type C virus. Suppression of GS antigen expression in LC × B10 hybrids was clearly correlated with a suppression of infectious type C virus production.

Observation of (LC × B10) F₁, F₂ and backcross progeny into old age is now under way to determine if resistance to lymphoma, epithelial tumours, and paralysis will occur in those hybrids segregating for lack of GS antigen and infectious virus expression.

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Second genetic locus in the HLA region for human B-cell alloantigens

THE ability to separate human peripheral blood T and B lymphocytes has allowed serological identification of specific B cell antigens, and several lines of evidence indicate that these antigens are controlled by a gene or genes of the HLA genetic region¹. Van Rood *et al.* presented evidence for an antigen which seems to be associated with, or closely linked to, the

locus responsible for mixed lymphocyte stimulation (HLA-D)². Our results in family studies suggest that there are a number of specific B-cell alloantigens that are controlled by genes linked closely to genes controlling the expression of HLA alloantigens^{3,4}.

B-cell alloantigens have been defined in murine systems and have been designated Ia antigens, which seem to be controlled by genes mapping within the H-2 complex⁵. At least three genes have now been identified controlling the different Ia antigens⁶. If the genetic region controlling cell-surface antigens on lymphocytes is analogous in the murine and human species, it seems probable that several genes might control the expression of human B-cell alloantigens. The results, presented here, of studies on B-lymphocyte antigens in a family where one member was an HLA recombinant provides evidence that such is the case.

We used human sera containing antibodies detecting B-cell specific antigens, obtained from multiparous women of a religious sect, the Amish, who, for cultural and religious reasons, live in rather isolated communities and, as a result, marry exclusively within the community. Peripheral blood lymphocytes were obtained from the father, mother and six children from the Amish family under study.

T and B lymphocytes were separated as previously described⁴ and tested by cytotoxicity with 34 antisera that were known to detect specific B-cell antigens and which did not react with HLA alloantigens. Briefly, this technique for separation and typing is as follows. Peripheral blood lymphocytes were isolated from heparinised whole peripheral blood by sedimentation with Ficoll-Hypaque, macrophages were depleted with carbonyl iron, and 2×10^6 lymphocytes were dispensed into microtitre plates that had been coated with antigen-antibody complexes. The lymphocytes were allowed to settle, resulting in adherence of the cell population bearing Fc receptors. The plates were then flooded, inverted, and the non-adherent cells recovered, concentrated, and 10^3 cells dispensed into microtitre plates for testing against the same sera that were used to test the adherent cell population. The adherent and the non-adherent cells were tested in triplicate with each of the 34 antisera and appropriate controls consisting of normal human sera and a known lymphocytotoxic sera. Whole rabbit serum was used as a source of complement.

Antiserum (5 μ l) was allowed to incubate with the cells for 1 h, the cells were washed with balanced salt solution containing 10% heat-inactivated foetal bovine serum and 5 μ l of complement added. After incubation the complement was removed, the cells washed, and 5 μ l of complement added for an additional 1-h incubation period. Complement was then removed and Trypan blue added. The reaction was deemed cytotoxic positive when > 30% of the cells (over that seen in the negative controls) incorporated Trypan blue. The background in negative controls was within the range 0-10% cells stained.

The results of the B-lymphocyte typing in this family are shown in Table 1 together with the HLA genotype of each family member. The serum reactivity reported here showed exclusive reactivity with B cells and no activity against the non-adherent or T-cell population. All the antisera can be assigned to HLA haplotypes and seven can be assigned to genetic sub-regions within the haplotype. Serum 640 was the unique marker for the maternal 1-8 haplotype inherited by sib 5. Sera 289 and 43 reacted against markers for the maternal 3-7 haplotype inherited by sibs 1, 2, 3, 4 and 6. Sera 35, 76, and 177 defined the 1-12 paternal haplotype inherited by sibs 3 and 4 and sera 590, 67, 322, 251 and 370 were directed against the 9-27 haplotype inherited by sibs 1, 2 and 5. Sib 6 was known from previous HLA typing to be a recombinant between the HLA-A and HLA-B loci. His cells were reacted against by sera 289 and 43, and by 35, 76, 322, 251 and 370. The reactions of 289 and 43 are clearly against the 3-7 haplotype. Reactions of sera 35 and 76 are shared with those sibs possessing HLA-A2 and are thus directed against an HLA-A region product. Sera 322, 251

Table 1 B lymphocyte typing in HLA recombinant family

Parents								
Mother				Father				
HLA type	1-8	3-7		2-12	9-27			
Serum reactivity	640	289		35	590			
with assignment	—	43		76	67			
to HLA haplotype	—	—		177	322			
					251			
					370			
Children								
	1. Edn		3. Erv		5. Nel		6. Rom	
	2. Erm		4. Ida				(Recombinant)	
HLA type	3-7	9-27	3-7	2-12	1-8	9-27	3-7	2-27
Serum reactivity	289	590	289	35	640	590	289	35
with assignment	43	67	43	76	—	67	43	76
to HLA haplotype	—	322	—	177	—	322	—	322
	—	251	—	—	—	251	—	251
	—	370	—	—	—	370	—	370

B lymphocyte typing in a family where one offspring was an HLA recombinant. Serological reactions with B cells are assigned to a particular HLA haplotype as reactions occurred in parents and offspring. The different sera reacting with these cells have been arbitrarily assigned numbers such as 640, 289, 43, and so on.

Table 2 HLA haplotypes* of three families and the reactions of B-cell antisera

[illegible]

*Three large families were tested for B lymphocyte antigens. Listed above are the HLA haplotypes found in these families and the reactions of their cells with the B-cell typing sera.

and 370 react with the HLA-B region product as shown by reactions of sibs 1, 2, 5 and 6. Note that sib 6 is not reactive with sera 590 and 67 associated with the intact 2-12 haplotype. This failure to react is not an artefact, as shown by absorption studies. Sera 177, 590 and 67 were absorbed with cells from sib 6 and retested against cells from the father. All remained fully active, showing that the antigens were indeed absent from the recombinant.

This shows clearly that the B-cell antigens are coded for by at least two genetic loci, one associated with the HLA-A portion of the haplotype and one with the HLA-B portion. This family study is not informative as to the precise location of the genes within the HLA-A and HLA-B regions. We can, however, be fairly sure that the genes for B-cell antigens are separate from the determinants for HLA-A and HLA-B from three lines of evidence. First, the sera react only against B cells; their failure to react against T cells argues against their being antibodies to the A, B, or C loci of HLA. Second, serum 590 was produced in a woman HLA 3-7/28-14 against a husband 3-7/3-BW55, so cannot react with HLA-A9, and a similar argument can be made for antiserum 67. The serum donor was 2-BW35/28-27 and her husband 2-7/11-27. Third, when tested on a random panel of B cells from subjects characterised for HLA, the antisera did not correlate with the distribution of HLA specificities. Further, when the reactions of the sera were examined in other families, different patterns of reactivity were observed. For example, in one family 590 reacted together with sera 177 and 176 to define the 2-7 haplotype in a group of nine sibs and in another family serum 177 reacted with four sibs inheriting the 9-BW15 haplotype whereas 590 reacted

with five sibs inheriting the 29-BW40 haplotype (Table 2). Thus not only do the different anti-B-cell sera sort with different HLA haplotypes in different families, they also seem to detect different B-cell specificities.

The present evidence indicates that there is a minimum of two loci for B-cell antigens within the HLA region. From the reactions of HLA haplotype-associated B-cell antisera in different families it seems probable that this is a minimum estimate and that the number of genetic loci may be considerably higher.

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Electron transfer across membranes using vitamin K₁ and coenzyme Q₁₀ as carrier molecules

CONTINUOUS redox processes have been effected between aqueous reactants, separated by liquid membranes in which coenzyme Q, or vitamin K, is present as a carrier molecule. These membranes show chemical specificity and function which relate closely to certain biological electron transfer processes.

The neutral lipid, coenzyme Q, is an essential link in the electron-transfer chain in mitochondria, and vitamin K may have a similar function in chloroplasts and certain bacteria.

We investigated the conditions in which these amphipathic quinone lipids may act as electron transfer agents, using solutions of vitamin K₁ and coenzyme Q₁₀ in hydrocarbon solvents as liquid membranes. These bulk membranes, being less dense than water, separated aqueous reducing agents and potentially reducible substrates (Fig. 1). The membranes may be considered to be composed of three parts; two interfacial monolayers, in which hydrophilic quinone or quinol groups are present at the aqueous interfaces, and the bulk hydrocarbon solution phase. Since there is a dynamic equilibrium between the monolayers and the bulk membrane phase, molecules reduced on one side may be transferred by diffusion to the opposite interface where they may reduce the aqueous substrate (A in Fig. 1). When both reductant and substrate are insoluble in the membrane phase each quinol (reduced) molecule may act as a carrier for two electrons and two protons. Electro-neutrality is thus maintained in both aqueous phases and a continuous process of electron transport may be maintained.

The normal reduction potentials (E'_0) for both vitamin K₁ and coenzyme Q₁₀ at pH 7 are close to 0 V (ref. 1) and so they are obtained in their oxidised forms in atmospheric conditions. To establish the requirements for a biphasic reduction, hexane solutions of quinone were equilibrated in oxygen-free conditions with various common aqueous reducing agents which had been shown to have no solubility in the hexane phase. Vitamin K₁ was reduced by only two reducing agents, methyl viologen and reduced flavin mononucleotide, FMNH₂, (a prosthetic group of flavoproteins) both in phosphate buffer at pH 7. Coenzyme Q₁₀ was less specific and also was completely reduced by aqueous dithionite and borohydride ions. For vitamin K₁, particularly, it seems that one-electron transfer agents are required. In support, electron spin resonance (ESR) spectra taken at the aqueous-liquid membrane interface during reduction with methyl viologen showed the presence of semi-quinone radicals as transient species for both quinones.

Of particular relevance to the role of coenzyme Q in natural membranes were the observations that, although aqueous solutions of NADH and NADPH were unable to reduce the

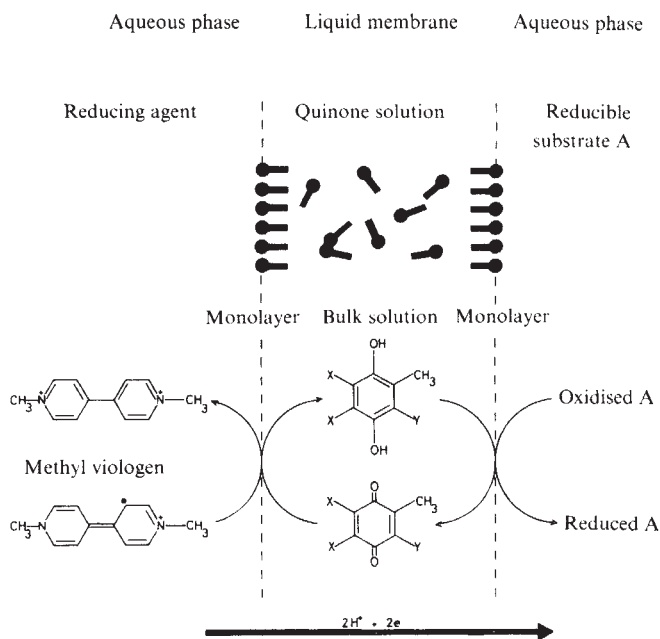


Fig. 1 Diagrammatic representation of the aqueous membrane model.

quinone directly, they may do so indirectly by a stepwise reduction involving FMN as an essential intermediate. These compounds, with their associated enzymes, have been shown to act in this sequence in the respiratory chain. Our results therefore indicate that limiting kinetic factors intrinsic to the electron-transfer process within the mitochondrion are already present in this simple analogue.

To establish continuous electron-transfer processes, experiments were conducted in an H cell, in which the quinone-hexane solution formed a liquid bridge between the two aqueous phases in the lower limbs of the cell. For convenience, reduced methyl viologen in phosphate buffer (pH 7) was used as reductant and various substrates were tested for reactivity. The apparatus was flushed with nitrogen and sealed. Equilibrations were conducted at 25 °C and the cell was vibrated gently to facilitate mixing in each of the three phases. Results, summarised in Table 1, show that a number of common redox indicators, used in biological studies on the respiratory chain, are completely reduced. Coenzyme Q₁₀ reduced ferric ion in acidic conditions and also iron III *o*-phenanthroline complex, which was reduced to ferroin. That cytochrome *c* was also completely reduced, indicated that these liquid membranes may be a suitable model for certain steps in the respiratory chain. Protonated molecules, such as reduced methylene blue, or protonated, oxidised, DCIP (the latter present to only 5% at pH 7) are soluble in hexane and are transported across the membrane. In mitochondria, this would provide a mechanism

Table 1 Tests of the reducing abilities of reduced vitamin K₁ and coenzyme Q₁₀ with a variety of common substrates

Substrate	E'_0 (ref. 1) (V)	E_0 (ref. 2) (V)	Reduced vitamin K	Reduced coenzyme Q	Comments
2,6-Dichlorophenol-indophenol	0.217		+	+	Back transfer of protonated (red) form
Indophenol	0.228		+		
Methylene blue	0.011		+	slight	Back transfer of reduced (leuko) form
Thionine	0.063		+	—	
FeCl ₃		0.77		+	
(Fe(<i>o</i> -phen) ₃)Cl ₃	1.12		+	+	Back transfer of uncomplexed <i>o</i> -phenanthroline
Cytochrome <i>c</i>	0.26		+	+	

The pH was maintained at 7 for each aqueous substrate except ferric chloride (which was in a solution of 0.1 M HCl) and the concentrations of the test solutions ranged from 0.01–0.10 mM.

of selective transport of molecules made lipid soluble by reduction or protonation.

Preliminary results have been obtained for the kinetics of reduction of aqueous substrates. The same H-cell format was used with the lower limbs (containing aqueous phases) replaced by optical cells. These aqueous limbs were stirred magnetically and turbulence and mixing of the quinone membrane solution obtained by gently rocking the whole apparatus. Tests showed that the rate of reduction of substrate was independent of whether or not the quinone was fully reduced at the start of the experiment. This confirmed that reduction by methyl viologen was rapid and that adequate agitation of bulk membrane had been obtained. Rates of transfer were insensitive to tenfold reduction in quinone concentration in the membrane, indicating a saturation effect typical of carrier-mediated transport. The reduction kinetics of methylene blue were first order with respect to the oxidised form and varied with stirring. These results are compatible with a diffusion-controlled mechanism, dependent only on the rate of diffusion of oxidised substrate from the bulk aqueous phase to the membrane interface. Electron transfer may thus be very rapid in this case.

Bimolecular lipid membranes have not been obtained, but stable thicker membranes $\sim 1 \times 10^2$ – 3×10^2 nm have been formed in Teflon orifices using decane solutions of both quinones. These have been shown to function in the same way as the bulk membranes described above.

Apart from the obvious biological implications, such membrane systems provide a new sensitive method for the preparation and characterisation of solutions of reduced or oxygen-sensitive compounds, without the added complication of chemical contamination (other than protons required to ensure electroneutrality). In particular, solutions of pure reduced cytochrome *c* and of the very oxygen-sensitive reduced vitamin K_1 in hexane were obtained in this study. The latter, previously uncharacterised, exhibited an intense maximum at 245 nm ($\epsilon = 44,500 \text{ l mol}^{-1} \text{ cm}^{-1}$) and a broader weaker band at 325 nm.

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Effect of experimental diabetes and insulin on phosphorylation of rat liver ribosomal protein S6

ONLY one rat liver ribosomal protein (S6) is phosphorylated *in vivo*¹. During hepatic regeneration the phosphorylation of S6 is increased by an order of magnitude, and derivatives are generated (in some experiments as many as five) which contain increasing numbers of phosphoserine residues¹. Very similar changes in the phosphorylation of S6 are caused by administration to animals of glucagon or cyclic AMP (A.M.G. and I.G.W., unpublished). Indeed, a number of hormones including adrenocorticotrophic hormone (ACTH)² and thyroid hormone³ increase the phosphorylation of ribosomal protein, although in the latter instances, the identity of the phosphoprotein has not been established. These observations have led us to consider the possibility that cyclic AMP is the mediator of the phosphorylation of

S6 (ref. 4). The concentration of cyclic AMP in the liver is increased in diabetic animals and restored to normal by insulin⁵, although it is still moot whether the effect of the hormone on the level of the cyclic nucleotide accounts for all (or even any) of its actions on hepatic cells⁶. We have tested the effect of experimental diabetes and of insulin administration on the phosphorylation of rat liver ribosomal protein: the phosphorylation of S6 was markedly increased by diabetes, and reduced towards the normal level by insulin administration.

Diabetes caused a 3.5-fold increase in the ³²P-radioactivity associated with liver 80S ribosomes (Table 1); the administration of insulin (5 units per 100 g body weight) to diabetic animals reduced the radioactivity associated with the particles by about one-half in 90 min; phosphorylation, however, was not restored to normal during that period (Table 1). Not all of the radioactivity associated with liver ribosomes after administration of ³²P-orthophosphoric acid to rats is in ribosomal protein¹. To test whether diabetes and insulin had actually affected the phosphorylation of ribosomal protein, subparticles were prepared from the ribosomes and the protein was extracted and the specific radioactivity determined. Diabetes increased the phosphorylation of the protein of the small ribosomal subunit by more than 3.5 times (Table 1); insulin administration decreased the specific ³²P-radioactivity of the protein of the 40S subparticle by somewhat less than one-half (Table 1). The effect of diabetes and insulin on phosphorylation of the small subunit protein can account for the alteration in radioactivity associated with 80S ribosomes. Very little radioactivity was in the protein of the large subunit and it was not appreciably changed by diabetes or insulin (Table 1). We have found¹ that phosphorylation of rat liver ribosomal protein *in vivo* is entirely in the 40S subunit; the radioactivity apparently associated with the large subunit is actually due to contamination with a small amount of 40S subparticles¹.

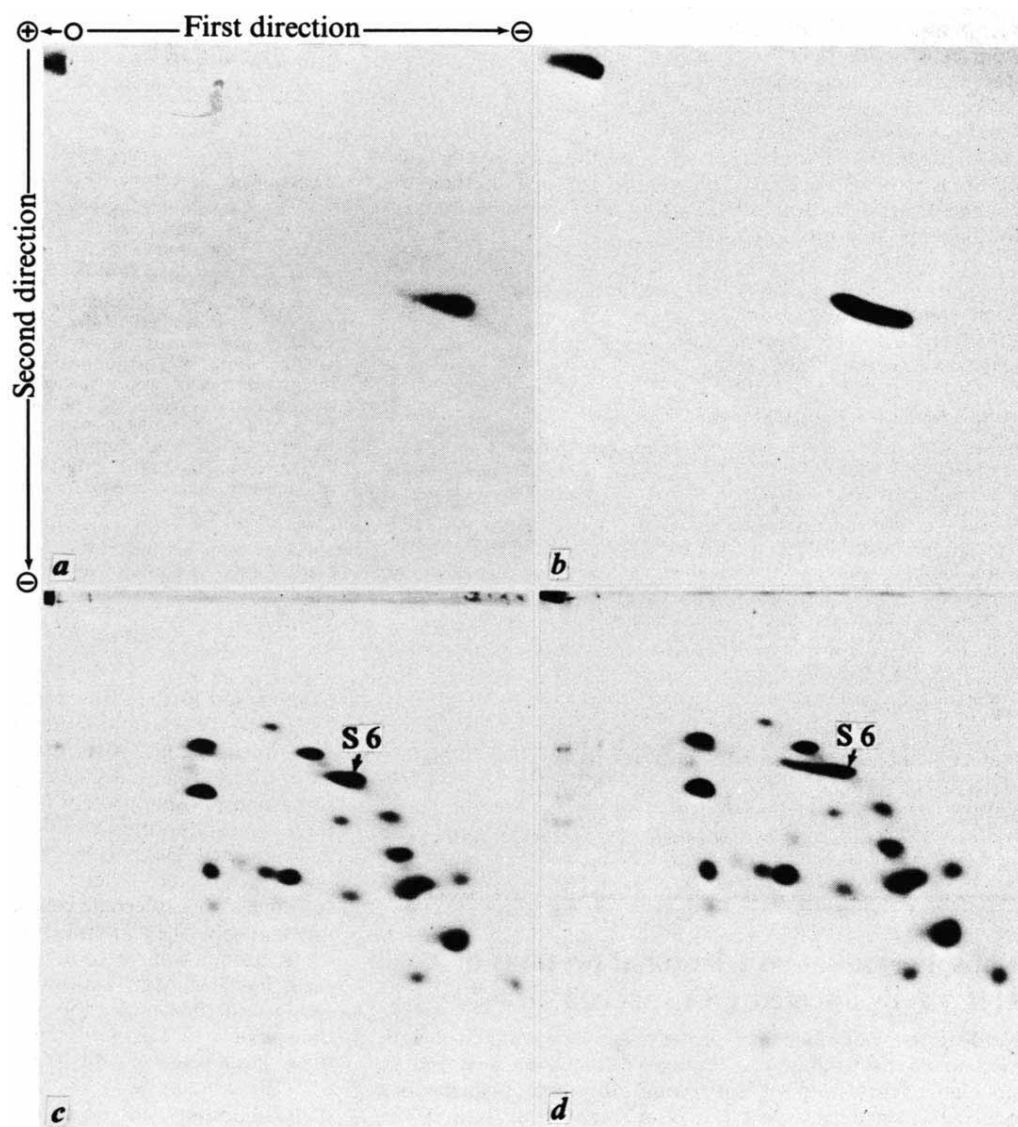
All of the radioactive phosphate incorporated into rat liver ribosomes *in vivo* is found in S6 (ref. 1). It seemed likely, therefore, that it was the phosphorylation of S6 that was affected by diabetes and insulin. To test the possibility the proteins of the 40S subunit were separated by two-dimensional polyacrylamide gel electrophoresis and radioautographs made of the gels (Fig. 1). 40S ribosomal subunits from animals that had received ³²P-orthophosphoric

Table 1 Incorporation of radioactivity from ³²P-orthophosphoric acid into rat liver ribosomes and ribosomal subunits

Source of liver ribosomes	³² P-radioactivity		
	80S	40S	60S
	(c.p.m. per A_{260}) (c.p.m. per μg protein)		
Normal	850	1.7	0.9
Diabetic	3,120	6.5	1.1
Insulin-treated diabetic	1,620	3.6	1.0

Male Holtzman rats (140–160 g) were starved for 24 h and injected intravenously with 65 mg per kg body weight of streptozotocin (lot no. 10518-GGS-37; provided by Dr W. E. Dulin, Upjohn) freshly dissolved in citrate buffer (pH 4.4). Control rats received the same volume of citrate buffer alone. Three days (68 h) after treatment with streptozotocin the fasting blood glucose levels were higher than 250 mg 100 ml⁻¹. The diabetic animals were at that time divided into two groups of two or three rats each: one group received intraperitoneally 5 U crystalline porcine insulin (lot no. 615-063-10, containing 0.002% glucagon; provided by Dr O. Behrens, Eli Lilly) per 100 g body weight in saline. Control animals received a similar volume of saline. Animals (control, diabetic and insulin-treated diabetic) were injected intraperitoneally with 3 mCi ³²P-orthophosphoric acid (carrier-free, in 0.02 N HCl, 285 Ci mg⁻¹, from ICN). Radioactive isotope was administered 90 min after animals received insulin or saline. All of the animals were decapitated 20 min after administration of the isotope. Livers were excised and ribosomes⁷ and ribosomal subunits⁸ prepared. Radioactivity in 80S ribosomal particles or in protein extracted from ribosomal subunits⁹ was determined as described previously¹. Concentration of protein in a sample was assessed¹⁰ using bovine serum albumin as a standard.

Fig. 1 Two-dimensional electrophoretograms and autoradiographs of the proteins of liver 40S ribosomal subunits. Rats were made diabetic and administered ^{32}P -orthophosphoric acid according to the protocol given in Table 1. Protein was extracted from the small ribosomal subunit⁹ and separated by two-dimensional polyacrylamide gel electrophoresis^{1,11,12}. Autoradiographs were made of the gels: *a*, protein from control animals; *b*, from streptozotocin diabetic animals. Electrophoretogram *c* is 40S ribosomal subunit protein from control animals; *d*, from diabetic animals. Enlargement of the autoradiographs is not the same as that for the electrophoretograms.



acid, but no other treatment, had a single radioactive phosphoprotein (Fig. 1*a*). Comparison of the autoradiograph (Fig. 1*a*) and the stained two-dimensional gel (Fig. 1*c*) established that the protein was S6. Diabetes seemed to increase the specific radioactivity of S6 (Fig. 1*b*), although one could not be sure of that from the autoradiograph alone.

It was certain, however, that diabetes had changed the conformation of the protein. The zone containing S6 on the stained two-dimensional gel was elongated with a tail extending towards the anode (Fig. 1*d*); several (perhaps, as many as four) distinct derivatives of S6 could be discerned, each more negatively charged. Similar changes have been shown before¹ to be the result of phosphorylation of increased numbers of serine residues in the protein. The autoradiograph (Fig. 1*b*) conformed to that interpretation; the intensity of the zone described a gradient of specific radioactivity that was greatest in the anodal portion and least where one would expect the normal S6. Similar changes in S6 occur during hepatic regeneration¹, as a result of the inhibition of protein synthesis with puromycin or cycloheximide⁴, and after administration of glucagon or cyclic AMP (A.M.G. and I.G.W., unpublished).

The increase in phosphorylation of S6 caused by diabetes cannot have been due solely to a change in the specific radioactivity of the intracellular pool of inorganic phos-

phate, or of ATP, as a result of a change in the transport of phosphate into hepatic cells. First, diabetes does not change the concentration of ATP in the liver¹². But more important, one can see the highly phosphorylated derivatives of S6 on stained gels after two-dimensional electrophoresis of ribosomal protein from diabetic animals; they were not seen when ribosomal protein from normal animals was analysed. The demonstration of the derivatives of S6 on gel plates does not depend on changes in specific radioactivity and thus authenticates the enhancement of ribosomal protein phosphorylation as a result of diabetes.

Whether the change in S6, caused by insulin deficiency, is the result of increased phosphorylation or decreased dephosphorylation is not known. That cyclic AMP causes similar changes (A.M.G. and I.G.W., unpublished), and that the hepatic concentration of the cyclic nucleotide is elevated in diabetes⁵, suggests that it is increased phosphorylation resulting from the activation of a protein kinase that is responsible. The changes in S6 are by no means specific for diabetes: similar alterations occur during hepatic regeneration¹ and after administration of glucagon or cyclic AMP (A.M.G. and I.G.W., unpublished); and probably after administration of other hormones as well^{2,3,14}. Insulin is the first agent, however, found to decrease phosphorylation of liver ribosomal protein. Insulin has also been reported to promote phosphorylation of

membrane and ribosomal protein in mammary gland stem cells¹⁵, although the phosphorylation of the ribosomes was not adequately characterised¹.

The functional significance of the phosphorylation of S6 is not known, although the possibilities have been discussed¹. Since the impact, if any, of the phosphorylation of S6 is not known, it is impossible to say whether the change bears a causal relation to the alterations induced by diabetes in protein synthesis¹⁶⁻¹⁸.

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Phosphorylation of ribosomal proteins in HeLa cells infected with vaccinia virus

MODIFICATION of ribosomal proteins has been suggested as a mechanism controlling protein biosynthesis¹. *In vivo* and *in vitro* phosphorylation of eukaryotic ribosomal proteins has been described and its possible functional relevance discussed²⁻⁵. Two-dimensional polyacrylamide gel electrophoresis has shown that only one, small ribosomal subunit protein, S6, is phosphorylated in rat liver⁶. The number of phosphoserine residues in this protein increases by an order of magnitude during liver regeneration⁶ and also after administration of inhibitors of protein synthesis⁷. In addition, when *Escherichia coli* is infected with bacteriophage T7, various host cell proteins, including some ribosomal proteins, are phosphorylated⁸. In our search for possible regulatory changes in the protein synthesising apparatus of virus-infected cells, we have now examined the phosphorylation of ribosomal proteins in HeLa cells infected with vaccinia virus.

Infection of HeLa cells with vaccinia virus caused a fourfold increase in the amount of radioactive phosphate incorporated into the ribosomal proteins of 40S ribosomes (Table 1). There is no difference in the specific radioactivity between 60S ribosomal proteins from uninfected and virus-infected cells. (Radioactivity in the total protein from uninfected 60S subunits cannot be due entirely to contamination of 60S subunits with 40S subunits. We are investigating whether it might be attributed to acidic phosphoproteins.) Subsequent treatment of ribosomal proteins with hot trichloroacetic acid (TCA) and organic solvents (to remove residual contamination due to RNA or phospholipids, respectively) did not substantially affect the difference in specific radioactivity between ribosomal proteins of 40S subunits from infected and uninfected cells.

The proteins of 80S ribosomes were extracted and separated by two-dimensional polyacrylamide gel electrophoresis in conditions which resolve only basic ribosomal proteins (Fig. 1).

Table 1 Incorporation of radioactivity from ³²P-orthophosphoric acid into the protein of ribosomal subunits from uninfected and vaccinia virus-infected HeLa cells

Experiment	Treatment	³² P radioactivity in ribosomal protein (c.p.m. µg ⁻¹)			
		Uninfected		Infected	
		40S	60S	40S	60S
a	Cold TCA	60	72	192	85
b	Hot TCA	42	45	192	69
c	Organic solvents	45	58	179	59

HeLa cells were infected with vaccinia WR virus at 1,000 virions per cell as described⁹. Infected and mock infected cells were suspended after virus adsorption in phosphate-free medium and labelled with ³²P-orthophosphate (50 µCi ml⁻¹). After 2.5 h of incubation, cells were washed and homogenised and ribosomal particles were pelleted¹⁰. The nascent polypeptides were released by treatment with 1 mM puromycin and ribosomes were separated by sucrose gradient centrifugation¹⁰ in 20 mM Tris-HCl, pH 7.5, 500 mM KCl, 3 mM MgCl₂, 5 mM β-mercaptoethanol. The ribosomal proteins were extracted, dialysed and lyophilised according to the procedure of Sherton and Wool¹¹. ³²P-ribosomal proteins were precipitated with (a) 10% TCA at 0 °C, the precipitate was collected after 30 min at 0 °C on glass fibre disks and washed with cold 5% TCA. b, As in a but heated for 20 min at 90 °C in 10% TCA before filtration. c, As in b except that additionally the samples on the disks were extracted with 10 ml of ethanol-ethyl ether (3:1) at 60 °C and as well as with 10 ml of ethyl ether and cold acetone.

Comparison of the stained gels revealed an additional protein located to the left (anodically) of protein S2 in ribosomes from virus-infected cells. Also, the density of staining of protein S2 from infected cells decreased. Autoradiographs of these gels show one phosphoprotein (P1) in uninfected HeLa cells and three phosphoproteins among the ribosomal proteins of virus-infected HeLa cells. Phosphoprotein P1 is common to both types of cells whereas P2 and P3 are only observed with ribosomes of infected cells. These phosphoproteins fulfil satisfactorily the criteria for ribosomal phosphoproteins¹³. (1) Ribosomes have been centrifuged twice through 0.5 M KCl, 0.003 M MgCl₂. (2) Phosphate is bound covalently to serine and threonine (not shown). (3) The covalently bound phosphate migrates with ribosomal proteins on gels. Further, the treatment with (4) hot TCA and (5) organic solvents showed that the contamination of ribosomal proteins with RNA or phospholipids is negligible.

To estimate quantitatively the amount of phosphate incorporated into individual proteins, proteins were cut out of the gel and ³²P radioactivity was determined. Table 2 shows that spot P1 is labelled in both types of cells whereas regions corresponding to P2 and P3 are not detectably labelled in uninfected cells.

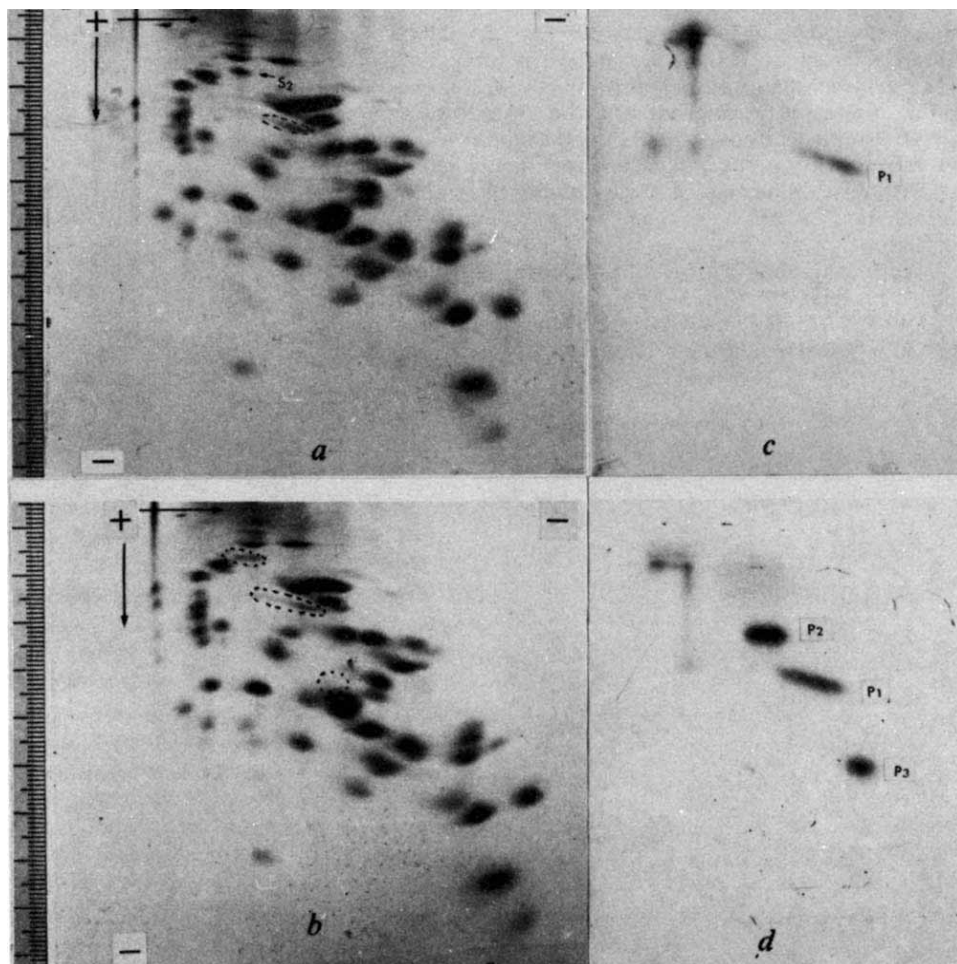
We have undertaken experiments to enumerate the ribosomal proteins of HeLa cells (D. Schiffmann and I. H., unpublished). According to this nomenclature of ribosomal proteins we have identified phosphoprotein P1 as S6 and P2 as S2. We assume that the additional protein spot observed in ribosomes from virus-infected cells (left of the normal position of S2) represents phosphorylated molecules of S2 protein. Its negative phosphate groups cause slower migration to the cathode in the first dimension. The lack of staining of gels in the radioactive

Table 2 Radioactivity in individual phosphoproteins separated by two-dimensional polyacrylamide gel electrophoresis

Protein	³² P radioactivity (c.p.m.) in protein from	
	uninfected cells	virus-infected cells
P1 (S6)	871	1,417
P2 (S2)	4	1,809
P3 (?)	0	544

The protein spots were cut out with a cork borer and their radioactivity was determined¹³. The radioactivity in the remaining ribosomal proteins (not shown) did not exceed 25 c.p.m. per spot. This blank value of 25 c.p.m. was subtracted from the values in the table.

Fig. 1 Two-dimensional electrophoresis of ^{32}P -ribosomal proteins from 80S ribosomes. Ribosomal proteins were prepared as described in the legend to Table 1. In (a) 820 μg of protein from uninfected cells and in (b) 820 μg of protein from virus-infected cells were separated by two-dimensional polyacrylamide gel electrophoresis¹², stained with Amido black and photographed. The circles indicate the radioactive areas from the autoradiographs. c, Autoradiograph of the gel a; d, autoradiograph of the gel b. There are no further spots on the autoradiographs beyond the confines of the figure.



area of phosphoprotein P3 suggests a high degree of phosphorylation. Because a high degree of phosphorylation could significantly alter the mobility of a ribosomal protein, it is difficult to ascertain whether or not P3 is a phosphorylated ribosomal, a non-ribosomal cellular or a viral protein.

The mechanism by which ribosomal phosphoproteins arise is not known. Two different reactions, phosphorylation by a kinase and dephosphorylation by a phosphatase, have been considered in the case of protein S6 from rat liver ribosomes⁷. A further possibility might be the phosphorylation of ribosomal proteins by a virion-associated protein kinase, such as that described in vaccinia virions¹⁴ and suggested to be involved in the regulation of host cell functions¹³. Finally, intracellular concentrations of cyclic AMP might be altered in infected cells and consequent effects on the phosphorylation of ribosomal proteins cannot be ruled out.

We cannot say whether the phosphorylation of ribosomal proteins is correlated with functional changes of the translational apparatus caused by the virus. It is tempting to speculate that the phosphorylation of ribosomal proteins is related to the switch off of host cell protein synthesis in virus-infected cells or to the specific selection of viral and cellular mRNAs for translation.

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Evidence that mutations in the *suA* polarity suppressing gene directly affect termination factor rho

THE *suA* mutation in *Escherichia coli* is defined by its suppression of the polar effects of nonsense mutations on distal genes¹. Richardson *et al.*² discovered that two independent mutations in the *suA* gene affect the transcriptional termination factor rho. This suggests² a mechanism for polarity: as long as ribosomes progress along the nascent message, transcription proceeds; but when ribosomal progress is blocked, exposure of the distal RNA somehow causes rho to terminate transcription prematurely. As others have noted³⁻⁶, the involvement of rho in translational polarity could explain several puzzling observations concerning the control of both transcription and translation. Richardson *et al.*² proposed that rho may be the product of the *suA* gene. They hesitated to draw that conclusion, however, because they found

a rather high level of rho activity, 9% of wild type, in a strain believed⁷ to contain an amber mutation in the *suA* gene (strain 2055). Either this amber lesion was extraordinarily leaky or, alternatively, the absence of *suA* gene function only partially and indirectly diminished the level of rho activity in the cell. The experiments reported here clarify this problem and argue that the transcriptional termination factor rho is indeed the direct protein product of the *suA* gene.

While examining the binding of bacterial and bacteriophage proteins to affinity resins which contain immobilised *E. coli* RNA polymerase⁸, I noticed that some preparations of immobilised polymerase retained the *E. coli* rho factor.

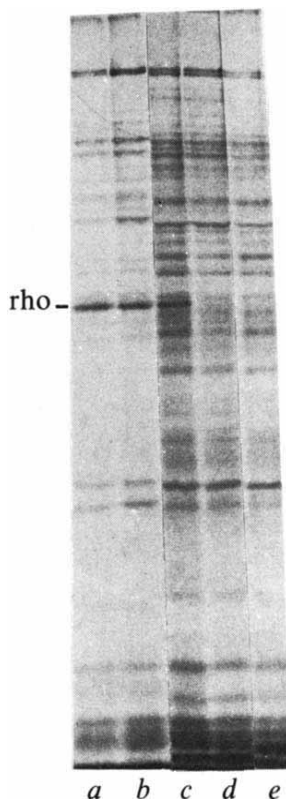


Fig. 1 Comparison of the rho polypeptide of *suA* strain 2055 with that of the *su*⁻ parent strain 2034. Radioactive cell extracts were prepared by freeze-thaw lysis, digestion of nucleic acids with DNase I and micrococcal nuclease, and brief centrifugation to remove debris (for details, see ref. 8). Extracts (25 µl, corresponding to 1 ml of original cell culture) were applied to affinity columns containing roughly 5 µg of immobilised RNA polymerase. The RNA polymerase holoenzyme used in the affinity resin had been purified through step V of the procedure of ref. 11 and then sedimented down a low salt glycerol gradient; the resulting enzyme is 90–95% pure, as judged by SDS-polyacrylamide gel electrophoresis. The preparation of the affinity resin and the microchromatographic procedures used have been described⁸. The figures show autoradiograms of the 1 M NaCl column elutions analysed on SDS-10% polyacrylamide slab gels⁸. The autoradiograms, using Kodak single-coated blue-sensitive X-ray film, were exposed for 10 d for samples *a* and *b*, and for 7 d for samples *c*–*e*. *a*, A culture of *su*⁻ 2034, grown at 37°C to $A_{550} = 0.60$ in minimal medium, was pulsed for 7 min with 100 µCi ml⁻¹ ³⁵S-methionine, chased briefly (3 min) with excess non-radioactive methionine, and collected on ice⁸. *b*, A parallel culture of *suA* 2055 was treated as above. *c*, A culture of 2034 was grown in minimal medium containing 4 µg ml⁻¹ ³⁵SO₄²⁻ (New England Nuclear Corp.), 350 µCi ml⁻¹. At $A_{550} = 0.30$, non-radioactive SO₄²⁻, methionine and cysteine were added in excess to prevent further incorporation of label; the chased cells were then grown for one further generation and collected. *d*, A parallel culture of 2055 was treated as above. *e*, Immediately before loading the affinity resin with a 25 µl sample of the 2034 extract described in *c*, the column was first saturated for rho by the addition of 3 µg of purified rho factor (given by R. Maurer, prepared by the method of Roberts¹²).

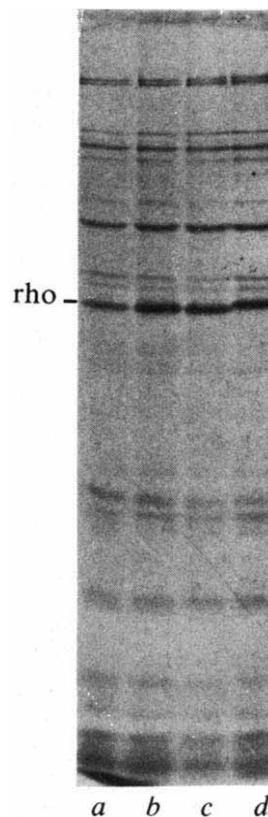


Fig. 2 Analysis of the rho proteins of polarity suppressor strains isolated by M. Malamy. Procedures were as described in Fig. 1. The radioactive pulse was for 7 min, followed by a 3 min chase. Affinity columns contained 15 µg RNA polymerase, and the final autoradiogram was exposed for 2 d. *a*, RV NaI, the *su*⁻ parent; *b*, RV NaI *su*¹¹⁵; *c*, RV NaI *su*²¹⁸; *d*, RV NaI *su*²²¹.

Subsequent experiments have shown that the ability to bind the rho protein is not an intrinsic property of immobilised RNA polymerase, but derives from the presence of a contaminant (possibly rho itself) in some preparations of the polymerase holoenzyme. I have, nevertheless, used these affinity resins to examine the rho protein of several *E. coli* strains. Making use of a slight difference in the molecular weights of the rho polypeptides of strain K12 and B/r, I mapped the rho gene near *ilv* at 75' on the *E. coli* genome⁹. (The observations reported here, in conjunction with the experiments described in ref. 9, make it almost certain that I have identified the structural gene for rho, and not some strain-specific modifier of the rho protein.) Pl co-transduction experiments showed the *rho* gene to be tightly linked to the *suA* locus (see ref. 9); and I was thereby led to examine the rho protein of several polarity suppressor strains.

To observe the rho polypeptide, I prepared crude cell extracts from ³⁵S-methionine pulsed cultures using freeze-thaw lysis and mild nuclease digestion (see the legend to Fig. 1 for details); the extracts were then applied to affinity columns which were subsequently eluted with a 1 M NaCl buffer. Figure 1 shows the sodium dodecyl sulphate (SDS)-polyacrylamide gel profiles of several of these column elutions. The polarity suppressing mutant 2055, the presumed *suA* amber^{2,7}, produces a rho polypeptide of normal molecular weight in amounts comparable with that made by the (*su*⁻) parent strain, 2034, during this pulse (compare columns in Fig. 1*a* and *b*). If the radioactive labelling protocol is changed, however, so that the cells are chased for one generation before collection, then the rho lesion in mutant 2055 becomes apparent: the columns in Fig. 1*c* and

d show that the rho protein of the parent strain is relatively stable during the *in vivo* chase, but the rho polypeptide of the polarity suppressed strain can no longer be detected after this treatment. The final column in Fig. 1e demonstrates that the autoradiographic band designated as rho represents this protein: in this experiment, previous addition of excess purified rho to the affinity resin totally and specifically overcame the binding of the radioactive rho protein in the 2034 extract.

The observation of wild-type levels of the full-sized rho polypeptide in pulsed extracts of *suA* mutant 2055 is not compatible with the assumption that 2055 harbours an amber mutation in the *rho* gene. On the contrary, the data of Fig. 1 suggest that 2055 may be a missense mutation resulting in an unstable rho protein. Genetic experiments confirmed that the *suA* phenotype of 2055 is not (currently) due to an amber mutation. Following the method which Morse and Guertin⁷ used to detect the original amber mutation, I introduced the *su3* amber suppressor into strain 2055 (of genotype⁷ *W3110 tryp E⁻ochre 9851, leu⁻amber 277, suA 120*) with $\phi 80\text{psu3}$, selecting *leu⁺* lysogens and then scoring for the continued expression of the *suA* allele. Each colony tested (22 independent isolates from the 2055 stab) continued to express its *suA* phenotype even in the presence of the *su3* amber suppressor. Examination of other strains presumed to carry the *suA* amber allele (2054 from D. Morse, and CU111 (a derivative of 2054) from H. Umbarger¹⁰) revealed no cells with true *suA* amber mutations. D. Morse and J. Richardson (personal communications) confirm that now the *suA* phenotype of these strains is not the result of an amber mutation in that locus. Since the *suA* lesion in strain 2055 is a missense mutation producing an unstable rho protein, the 9% level of activity detected by Richardson *et al.*² is in complete agreement with the contention that rho is the primary gene product of the *suA* locus.

I have screened the rho proteins of another set of polarity suppressed mutants, those isolated by M. Malamy (in preparation) for their effect on insertion mutations in the lactose operon¹³. The polarity suppressing mutations map at 75' and are presumed to lie in the locus originally designated *suA*. Figure 2 shows that three of these mutations do affect rho. In mutant 221, the rho polypeptide is of slower gel mobility (presumably greater molecular weight) than normal. The simplest interpretation of the 221 effect is that the *suA* mutation lies in the structural gene for rho. Each of the *suA* strains seems, by this method of detection, to overproduce the rho protein. Mutant 115, the strongest polarity suppressor in that collection (M. Malamy, personal communication), is the greatest overproducer. Densitometry of the autoradiograms indicates that pulsed cultures of strain 115 contain at least three times as much radioactivity in the rho polypeptide as the RV Nal parent strain. (Even after a one generation chase, strains 221 and 115 display at least as much radioactive rho as the parent strain.) It is surprising that these polarity-suppressing strains, presumed to contain diminished rho activity, seem to overproduce the rho polypeptide. This may be an indication of autoregulation of rho synthesis, conceivably the result of a rho-dependent attenuation of transcription¹⁴ of the *suA* gene.

The *suA* mutations described here affect rho in disparate ways: strain 2055 makes an unstable rho; other strains, especially *su¹¹⁵*, overproduce rho; and *su²²¹* apparently increases the molecular weight of the polypeptide. There are other mutations, linked to *ilv*, that affect specific termination processes. The mutants of Bertrand *et al.*¹⁴, which suppress attenuation of the tryptophan operon, also suppress polarity, but the sun mutants described by Brunel and Davison¹⁵, relieving the N requirement of phage λ , do not. The widely differing phenotypes of all these mutants are probably a consequence of the necessarily subtle nature of

mutations allowed in rho, reflecting the essential role that rho must have in bacterial transcription.

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Exhaustion and recovery of repair excision of *O*⁶-methylguanine from rat liver DNA

EVIDENCE continues to accumulate that the *O*⁶ position of guanine in DNA is a critical reaction site for the initiation of malignant transformation by monofunctional alkylating agents. *O*⁶-alkylguanine has been shown to be promutagenic and the relative extent of its formation by different compounds approximately corresponds to their carcinogenic potency¹⁻³. In contrast to 7-alkylguanine, which is the major reaction product in nucleic acids, *O*⁶-alkylguanine can be enzymically excised from DNA⁴⁻⁷ and the tissue-specific carcinogenic action of alkylating carcinogens seems to depend on the differential capacity of the various organs to actively remove this base from their DNA⁸⁻¹¹. The principal target organ in the carcinogenicity of *N*-methyl-*N*-nitrosourea (MNU) is the nervous system and it has been shown that *O*⁶-methylguanine (*O*⁶-meG) is removed much less rapidly from brain DNA than from that of any other rat tissue^{9,11}. The liver is not susceptible to the carcinogenicity of MNU (except after partial hepatectomy¹²) and this organ has been found to be most efficient in the repair excision of *O*⁶-meG (ref. 11). We have noted⁹, however, that the loss of *O*⁶-meG from liver DNA is influenced markedly by the dose of MNU administered; higher levels of *O*⁶-meG are less rapidly removed. We now present evidence that the excision repair system for *O*⁶-meG in rat liver can be overloaded with sublethal doses of MNU and related carcinogens, and requires several days to recover and restore its initial capacity.

In all experiments, female BD-IX rats (110-140 g) received an intravenous injection of 10 mg kg⁻¹ ³H-MNU and were killed 6 h later. Liver DNA was isolated and purine bases were separated by Sephadex G-10 chromatography of the acid hydrolysate¹¹. After injection of ³H-MNU alone *O*⁶-³H-meG present in liver DNA 6 h later amounted to 3.3 × 10⁻⁴ mol % of guanine (Fig. 1). When unlabelled MNU (70 mg kg⁻¹) was administered simultaneously with, or up to 12 h before, the injection of ³H-meG the amount of *O*⁶-³H-meG present 6 h later was 7-10 times higher, indicating that it was excised from DNA at a considerably lower rate. The excision capacity of the liver gradually recovered during longer pretreatment intervals but even 7 d after the injection of unlabelled MNU the initial rate

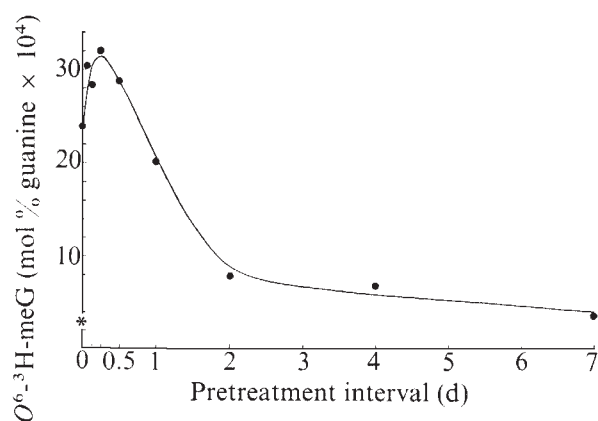


Fig. 1 Amounts of O^6 - 3H -meG present in liver DNA 6 h after an intravenous injection of 10 mg kg^{-1} 3H -MNU. Unlabelled MNU (70 mg kg^{-1}) was administered either simultaneously or at various times before injection of 3H -MNU (pretreatment interval). Asterisk on ordinate indicates amount of O^6 - 3H -meG present when no additional unlabelled MNU was administered. DNA was isolated by phenol extraction from the pooled livers of two rats and hydrolysed in 0.1 M HCl (37°C , 20 h). Purine bases were determined after chromatography on Sephadex G-10 columns ($1 \times 90 \text{ cm}$) eluted with $0.05 \text{ M ammonium formate}$ ($\text{pH } 6.8$). Extent of methylation was calculated from specific radioactivity of injected 3H -MNU ($12.5 \text{ mCi mmol}^{-1}$). For details see ref. 11.

of O^6 - 3H -meG excision was not yet fully re-established. The effect of pretreatment with various doses of unlabelled MNU on the amount of O^6 - 3H -meG persisting in liver DNA is shown in Fig. 2. In this experiment, a constant pretreatment interval of 3 h was chosen. The results provide no evidence for a threshold effect: even pretreatment with a dose of only 10 mg kg^{-1} MNU significantly increased the amount of O^6 - 3H -meG present 6 h after the administration of 3H -MNU.

To test the specificity of the pretreatment effect, sublethal doses of X rays and agents of various carcinogenic potency were applied (Table 1). Compounds known to produce a high extent of alkylation in the O^6 position of guanine (ethylnitrosourea, dimethylnitrosamine³) markedly reduced the excision of O^6 - 3H -meG produced by the subsequent injection of 3H -MNU. Methyl methanesulphonate was given at a dose (85 mg kg^{-1}) known to produce approximately the same total extent of alkylation of DNA as 70 mg kg^{-1} MNU¹³. The effect of methyl methanesulphonate pretreatment on the excision of O^6 - 3H -meG, however, was equivalent to a dose of only about 4 mg kg^{-1} MNU (Table 1, Fig. 2). This could be explained by the fact that 4 mg kg^{-1} MNU would be expected to produce approximately the same amount of O^6 -meG as 85 mg kg^{-1} methyl methanesulphonate¹⁴. At the same time, the results of methyl methanesulphonate

administration indicate that the pretreatment effect on O^6 -meG excision is indeed mediated by the production of O^6 -alkylguanine itself rather than alkylation products such as 7-alkylguanine. The other agents used for pretreatment (Table 1) are not known to produce O^6 -alkylation of guanine. The small effects observed after pretreatment with *N*-nitrosomorpholine, 7,12-dimethylbenz [*a*] anthracene and cyclophosphamide suggest that these agents may only to a limited extent produce DNA lesions which are recognised and repaired by the O^6 -meG excision system. Ethionine, like nitrosomorpholine a potent liver carcinogen, reacts with DNA to only a very small extent¹⁵ and was found to have no effect on the excision of O^6 - 3H -meG from liver DNA. X irradiation was the only pretreatment which caused a more rapid excision, although to only a small extent.

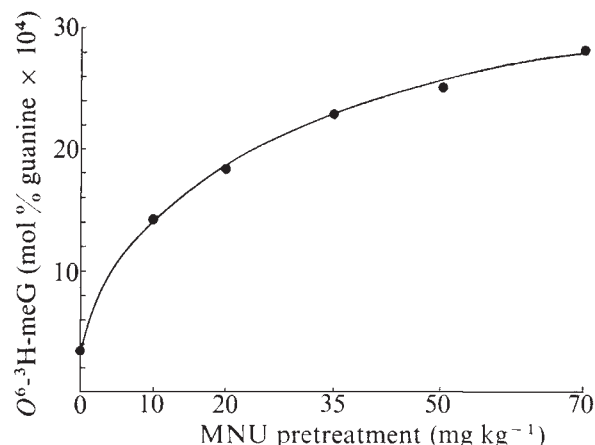


Fig. 2 Effect of various pretreatment doses of MNU on excision of O^6 - 3H -meG from rat liver DNA *in vivo*. Animals received 10 – 70 mg kg^{-1} unlabelled MNU. After an interval of 3 h, they were given 3H -MNU (10 mg kg^{-1}) and killed 6 h later. For experimental details, see Fig. 1.

The results obtained cannot be explained by an increase in the overall rate of alkylation attributable to the pretreatment since this would proportionally lead to an increase in alkylation at the 7 position of guanine. Only minor changes in the 7 - 3H -meG levels, however, were found: the decrease in the O^6 -meG excision was evident from an increase in both the absolute amounts of O^6 - 3H -meG and the O^6 -/ 7 - 3H -meG ratio (Table 1).

The present study shows that rat liver may efficiently excise O^6 -alkylguanine produced by a single dose of an alkylating carcinogen but that a second dose, given after an interval of less than about 12 h, produces additional DNA damage which is much less efficiently repaired. This may correspond to the observation that liver carcinogens in general do not produce tumours when given as a single

Table 1 Effect of pretreatment with various agents on excision of MNU-induced O^6 -meG from rat liver DNA

Pretreatment	Dose	Route	O^6 - 3H -meG*	Ratio O^6 -/ 7 - 3H -meG
Control	—	—	3.3	0.018
X irradiation†	400 R	—	1.8	0.011
DL-Ethionine	750 mg kg^{-1}	i.p.	3.2	0.023
Cyclophosphamide	90 mg kg^{-1}	i.v.	4.3	0.031
7,12-Dimethylbenz [<i>a</i>] anthracene	100 mg kg^{-1}	i.p.	6.4	0.029
<i>N</i> -nitrosomorpholine	50 mg kg^{-1}	i.v.	7.5	0.039
Methyl methanesulphonate	85 mg kg^{-1}	i.v.	10.4	0.054
<i>N</i> -ethyl- <i>N</i> -nitrosourea	120 mg kg^{-1}	i.v.	18.9	0.086
MNU (cold)	70 mg kg^{-1}	i.v.	28.3	0.120
<i>NN</i> -dimethylnitrosamine	20 mg kg^{-1}	i.p.	32.3	0.125

* O^6 - 3H -meG in liver DNA, expressed as $\text{mol \%} \times 10^4$ of guanine.

†Whole body irradiation using a ^{60}Co source.

Animals received an intravenous injection of 10 mg kg^{-1} 3H -MNU and were killed 6 h later. Pretreatment with equitoxic doses (approximately 50% of the acute LD_{50}) of various agents was carried out 3 h before injection of 3H -MNU. i.p., intraperitoneal; i.v., intravenous.

dose but are most effective after daily administration in the diet or drinking water¹⁶. The pretreatment scheme used in this study may be useful to test whether a carcinogen produces DNA lesions which saturate the O⁶-alkylguanine excision system by competing for the same repair enzymes.

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Altered C-terminal salt bridges in haemoglobin York cause high oxygen affinity

STUDIES of abnormal human haemoglobins (Hbs) with increased O₂ affinity have been particularly useful in developing detailed molecular interpretations of normal Hb function. A number of functionally interesting mutants are altered in the BHC region but only one variant, Hb Hiroshima (β146 His→Asp)^{1,2}, has been reported as having a substitution at the C terminus itself. We report here the structure and O₂ equilibria of Hb York, a new mutation at the β146 position, His→Pro, associated with increased O₂ affinity, decreased cooperativity and diminished Bohr effect.

Hb York was discovered during investigation of mild asymptomatic erythrocytosis in a 22-yr-old Caucasian woman. Preliminary measurements showed markedly increased whole blood O₂ affinity, suggesting the presence of an abnormal Hb. Although Hb electrophoresis on starch gel or on cellulose acetate at alkaline pH failed to reveal an abnormal band, two major components were separated on agar gel electrophoresis at pH 6.0 in 0.05 M citrate buffer³. Hb York migrated more rapidly towards the cathode than Hb A and constituted approximately 50% of total Hb.

Hb York was separated from Hb A on carboxymethyl-cellulose³, and was shown to be homogeneous on agar gel electrophoresis. The presence of approximately equal amounts of Hb A and York suggested that Hb York was a β mutant. The fractions containing Hb York in the CO form were concentrated by pressure ultrafiltration and dialysed exhaustively against 0.1 M NaCl.

Purified Hb York was converted to globin with cold acid acetone⁴. The abnormal β chain was isolated by the method of Clegg *et al.*⁵. Amino acid analysis of the HCl-hydrolysed β chain showed a low value for histidine and a high value for proline compared with β^A chains. The β^{York} chains were aminoethylated as described previously⁶ and digested with trypsin⁷. The tryptic peptides were chromatographed on Aminex A-5 (ref. 8), and the chromatogram appeared similar to that resulting from a tryptic digest of β^A chains except that the βT-15 (Tyr–His) was absent. Rechromatography on Aminex AG 50W-X4 of a fraction containing the βT-4 and an abnormal peptide gave a good yield of a peptide having the amino acid

composition shown in Table 1. This corresponds to the normal βT-14,15 peptide except that a histidine has been replaced by proline. This substitution was confirmed by analysis of the peptides resulting from partial hydrolysis of the βT-14, 15 peptide by thermolysin (Calbiochem, Crystalline B Grade)⁹. Analysis of the other tryptic peptides following HCl hydrolysis revealed them to have normal composition.

To distinguish between mutation of the β146 and β143 histidine residues, tryptic hydrolysis of the β chain after glycinamidation by the method of Carraway and Koshland¹⁰ was carried out. A normal βT-14 peptide was obtained in good yield, indicating that the mutation was at the β146 rather than the β143 histidine. The glycinamidated βT-15 (Tyr–Pro) peptide was not observed, presumably owing to the degrading effect of glycinamidation on tyrosine residues.

Carboxypeptidase A digestion of β^A and of β^{York} chains was carried out according to Ambler¹¹ in 2 M urea at pH 8.5 for 2 h at 37 °C. Although both Tyr and His were removed from the β^A chain, no amino acid residues were removed from β^{York}. Proline at the C terminus would be expected to resist the enzyme. Hb York is therefore β146 (His→Pro).

Functional studies of Hb York revealed a very high O₂ affinity and diminished Bohr effect and cooperativity. The log *P*₅₀O₂ of Hb York was 0.04 in contrast to 0.71 for similarly prepared Hb A, measured at 20 °C in 0.1 M phosphate, pH 7.0. The alkaline Bohr effect of Hb York was approximately 50% normal ($\Delta \log P_{50}/\Delta pH = -0.36$ compared with -0.65 for Hb A). Hill's 'n' for Hb York is 1.8 (± 0.2) compared with 2.5 for Hb A in these conditions, and did not change with pH. The effect of the organic phosphates, 2,3 diphosphoglycerate (DPG) and inositol hexaphosphate (IHP), on the O₂ equilibria of Hb York and Hb A is shown in Table 2. DPG and IHP are known to bind to the β chains and stabilise the quaternary conformation of deoxy Hb, thus lowering O₂ affinity. The changes of log *P*₅₀O₂ of Hb York produced by DPG and by IHP are similar to those observed with Hb A. In contrast to the diminution of Hill's 'n' to 1.6 observed with Hb A in the presence of IHP, the value for Hb York was increased from 1.8 to 2.2. The visible and ultraviolet spectra of Hb York were similar to those of Hb A.

Hb York may be contrasted with Hb Hiroshima (β146, His→Asp)². Both mutants have a similarly reduced Bohr effect owing to loss of the intrachain salt bridge between the β146 histidyl imino NH⁺ and the β94 aspartyl carboxylate group. The Bohr effect of des-(His-β146) Hb A has also been shown to be diminished¹³. The O₂ affinity of Hb York is,

Fig. 1 Model of C-terminal region of β chain of deoxy Hb York showing proposed salt bridge between β146 Pro carboxylate and ε-amino group of β144 lysine. Constructed from coordinates of horse deoxy Hb except for the inclusion of prolyl residue and rotation of lysine side chain.

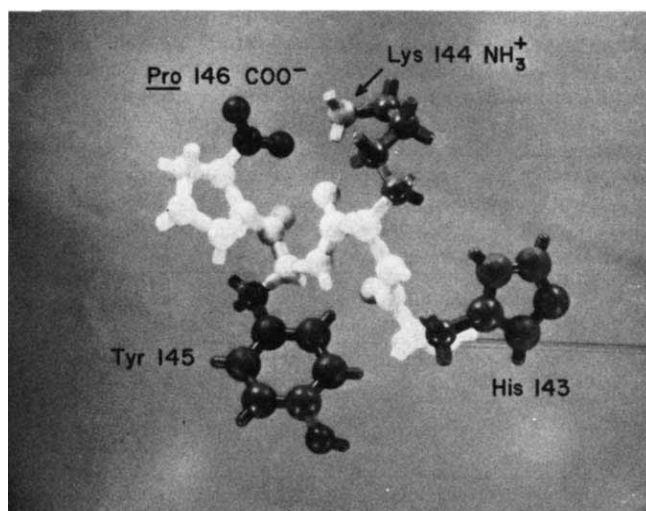


Table 1 Composition of C-terminal peptides of β chain of Hb York (amino acid residues per haem)

Amino acid	Tryptic β T-14,15	Thermolysin peptide from β T-14,15	Glycinamidated tryptic β T-14
Proline	1.0(0)*	1.0(0)*	—(0)*
Tyrosine	0.8(1)	1.0(1)	—(0)
Histidine	1.0(2)	0.9(2)	1.0(1)
Lysine	1.1(1)	1.1(1)	1.1(1)
Alanine	4.0(4)	1.0(1)	3.7(4)
Leucine	1.1(1)	1.0(1)	1.0(1)
Aspartic acid	1.1(1)	—	1.1(1)
Valine	3.0(3)	—	2.9(3)
Glycine	1.0(1)	—	1.0(1)

* Values in parentheses are those expected for corresponding peptides in normal Hb.

however, significantly higher than that of Hb Hiroshima or of des-(His- β 146) Hb A (Table 3). Hill's ' n ' is lower than that observed for either of these Hbs.

The mechanism for the abnormal O_2 affinity of Hb York may involve destabilisation of its ' T ' conformation. This destabilisation might be due to loss not only of the intrachain salt bridge between the β 146 His NH^+ and the β 94 Asp carboxylate but also to impaired formation of the interchain salt bridges between the β 146 carboxylate and α 40 Lys ϵ -amino group which is characteristic of the normal T conformation of deoxy Hb¹⁴. Hb Hiroshima does not lose the latter salt bridge². We constructed a model of the C-terminal region of the β chain of horse deoxy Hb, using energy refined coordinates supplied by M. F. Perutz. This model indicated that formation of the

salt bridges which contribute to the stabilisation of the deoxy conformation of Hb A.

Crystals of deoxy des-(His- β 146) Hb A have also been described¹⁵ as lacking the four β -chain salt bridges, yet the functional properties of this haemoglobin are more similar to those of Hb Hiroshima than Hb York. The modifications to the $T \rightleftharpoons R$ equilibrium of the des-(His- β 146) derivative seem to be more complex¹⁶ than in Hb York.

The loss of the four β -chain C-terminal salt bridges is not sufficient to prevent deoxy Hb York from entering the T conformation because it possesses a significant degree of cooperativity, because the effects of DPG and IHP on its O_2 affinity are similar to those for Hb A, and because the visible and ultraviolet spectra of deoxy Hb York are very similar to those of deoxy Hb A. The degree of destabilisation of the T conformation proposed for Hb York, however, would be greater than that occurring in Hb Hiroshima due to the loss of the interchain salt bridges. This additional destabilisation would account for the more pronounced abnormalities of O_2 binding of Hb York.

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Table 2 Effects of organic phosphates on O_2 equilibria* of Hb York and Hb A

	$\log P_{50O_2}$	Hill's n	$\Delta \log P_{50O_2}^\dagger$
Hb York			
'Stripped'	-0.21	1.8	—
0.2 mM DPG	-0.02	2.0	0.19
1.0 mM IHP	0.70	2.2	0.91
Hb A ‡			
'Stripped'	0.67	2.6	—
0.2 mM DPG	0.90	2.5	0.23
1.0 mM IHP	1.63	1.6	0.96

* O_2 equilibria determined at pH 7.0 and 20 °C using a modified³ technique of Benesch *et al.*¹². Samples were buffered with 0.05 M bis-Tris and contained 0.1 M chloride. Formation of methaemoglobin during these experiments did not exceed 5% of the total.

$^\dagger \Delta \log P_{50O_2} = \log P_{50O_2} - \log P_{50O_2}$ (Stripped Hb).

‡ Hb A was prepared by passing a haemolysate from normal human adult red cells through Sephadex G-25 equilibrated with 0.1 M NaCl.

β 146- α 40 salt bridge is seriously hindered in Hb York. The referee has kindly stated that "The reason why the salt bridge between the α -carboxyl of Pro- β 146 and Lys- α 40 is not formed is that the Pro is too close to the Cys- β 93." The β 146 proline residue and the side chain of the β 144 lysine can, however, be readily oriented (Fig. 1) so that an intrachain salt bridge is formed between the β 146 carboxylate and the ϵ -amino group of β 144 lysine. Formation of this salt bridge does not alter the position of the critical β 145 tyrosine. We suggest that this salt bridge would not be broken by transition to the ' R ' conformation on oxygenation and, therefore, would not contribute to the free energy of interaction. We thus propose that the deoxy conformation of Hb York lacks the four β -chain C-terminal

Table 3 Comparison of O_2 equilibria* of Hb York with Hb Hiroshima and des-(His- β 146) Hb A

	Hb York	Hb Hiroshima	des-(His- β 146) Hb A
$\log P_{50O_2}$	0.04	0.3	0.28
Hill's n	1.8	2.0	2.5
Bohr effect	Decreased	Decreased	Decreased
Reference	This paper	1	13

* Properties determined at or corrected to pH 7.0, 20 °C in 0.1 M phosphate buffer.

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matters arising

Weak interactions in the big bang

THE apparent arbitrariness of the numerical values assumed by the various constants of physical theory has from time to time led to the conjecture that these quantities may not be fixed numbers at all, but vary from epoch to epoch as the Universe evolves. Such a suggestion was advanced, on the basis of some discussion about gauge theories and spontaneous symmetry breaking, by Domokos, Janson and Kövesi-Domokos¹ in connection with the weak interaction coupling strength. They propose that this coupling strength was comparable with electromagnetism during the primordial fireball phase of the big bang (specifically, before $T \approx 10^5$ K). This is some 10^{10} times greater than at present, and its consequences are being investigated by the authors.

One consequence which they will discover is that, on general grounds, the lifetime of the neutron against β decay, $n \rightarrow p + e^- + \bar{\nu}$, would presumably be reduced by the same factor of $\sim 10^{10}$, making the half life of neutrons in the fireball phase a mere 10^{-7} s or so. The implications of this are profound. According to current models of the hot big bang, protons and neutrons are maintained in thermodynamic equilibrium by electron-positron processes until the end of the lepton era at ~ 1 s (10^{10} K). After this, the electron-positron pairs annihilate, and the neutron/proton abundance ratio is thereafter 'frozen in' until the temperature drops sufficiently low (about 10^9 K) for helium synthesis to occur. Essentially all the remaining neutrons are incorporated into helium (where they remain stable) after a few hundred seconds, which is well within the currently measured lifetime of the neutron.

Accepting the model of Domokos *et al.* would lead to the conclusion that the neutron abundance would not remain frozen at all during the intermediate cooling phase, but would decay to virtually zero long before nucleosynthesis could begin. The consequences of that would be an absence of neutrons in the universe—indeed, a universe composed entirely of hydrogen.

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DOMOKOS ET AL. REPLY—DAVIES' remark¹ concerning our recent letter² raises a relevant issue; in fact, we have been studying this problem for some time. If interpreted literally, our result may lead to the paradox pointed out by Davies. The careful reader will, however, have noticed that in order to illustrate the basic idea expressed in our letter (generation of a distinction between weak and e.m. interactions through a cosmological background) we took an extremely crude idealisation of an expanding universe. In fact, our input scalar curvature is: $R/(3p - \rho) = 0$ ($x < x_0$) and $R \sim x^{-3}$ ($x > x_0$). In such a universe the temperature drops abruptly from infinity to essentially 'zero' at x_0 . Hence, for $x < x_0$ there is no nucleus formation and no neutron decay either (due to an infinite time dilatation factor). All this can take place only for $x > x_0$. Then $\phi(x)$ rises very rapidly and gets close to its present value quite soon. Thus, even in this highly idealised model, there seems to be no qualitative contradiction with usual ideas of He formation. On the basis of computer experiments performed with more realistic models, we are now under the impression that the general shape of the curve $\phi(x)$ is correctly given by our model². Davies' observation imposes a consistency condition on more realistic model calculations: ϕ must come out to be substantially different from zero during the epoch of primordial He formation.

We thank P. C. W. Davies for informing us about his observations before publication. This research is being supported by the US Energy Research and Development Administration.

¹ Davies, P. C. W., *Nature*, 259, 157 (1976).

² Domokos, G., Janson, M. M., and Kövesi-Domokos, S., *Nature*, 257, 203–204 (1975).

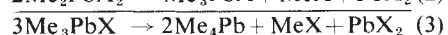
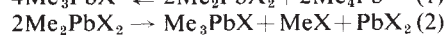
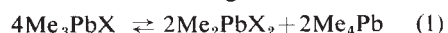
Methylation of organolead and lead(II) compounds to $(CH_3)_4Pb$ by microorganisms

RECENTLY evidence¹ showed that microorganisms can transform certain lead compounds into Me_4Pb ; addition of Me_3PbOAc to sediment samples apparently always increased the production of Me_4Pb . The addition, of lead(II) compounds did not, however, lead to definite effects, and with pure bacterial isolates no transformation of Pb^{2+} to Me_4Pb could be observed¹. Doubt has since been cast² on the significance of a microbiological conversion of Me_3PbOAc to Me_4Pb , preferring a chemical mechanism;

also alkylation of $Pb(NO_3)_2$ could not be detected (ref. 2).

Our studies of the behaviour of lead compounds in the presence of microorganisms show that Pb^{2+} can be biologically alkylated to Me_4Pb , and this helps to explain the formation of Me_4Pb from Me_3PbOAc in anaerobic systems.

In aqueous solution, alkyl lead compounds R_2PbX_2 and R_3PbX ($X = \text{anion}$) redistribute according to



reaction (2) is much faster than (1)^{3,4} and therefore Me_3PbX seems to be relatively stable at room temperature. The stoichiometry of its decomposition can be described practically by reaction (3).

In water samples (dilution water^{5,6}) seeded with microorganisms from an aerated aquarium, we observed, apart from the inhibition of biodegradation, that di-alkyl and tri-alkyl lead concentrations decreased considerably faster in both aerobic and anaerobic conditions than in sterile samples, and that the proportions of products no longer agrees with predictions from the above system of equations. After addition of Me_3PbCl to an anaerobic sample, less Pb^{2+} , but more Me_4Pb was formed than is required by reaction (3). Furthermore, the amount of Me_4Pb produced in a given time was considerably higher than from a sterile sample (by a factor of 5–10 in a week). Therefore the possibility that Me_4Pb was produced only by redistribution (reaction (1)), could be excluded, and it was an obvious assumption that Me_3PbCl and/or Pb_2^{2+} , formed according to reaction (3), had been alkylated by microorganisms to Me_4Pb .

We then investigated whether Pb^{2+} could be alkylated by microorganisms in the same anaerobic conditions. We inoculated water samples containing $Pb(OAc)_2$ in different low concentrations (Table 1) and sealed the flasks after flushing with N_2 . The analysis for Me_4Pb (the only volatile lead compound in the system) was carried out by slowly sweeping the gas phase above the solution with N_2 through an 0.2 N methanolic iodine scrubber solution⁷; the lead content was determined colorimetrically with 4-(2-pyridylazo)-resorcinol⁸. The experiments were reproducible, provided that the seed was not more than 6–7 weeks old.

We therefore conclude, that besides the formation of Me_4Pb by redistribution according to reaction (1), biological alkylation of Pb^{2+} must be considered as

¹ Domokos, G., Janson, M. M., and Kövesi-Domokos, S., *Nature*, 257, 203–204 (1975).

Table 1 Methylation of Pb^{2+} by microorganisms

Pb^{2+} Concentration* ($\mu g\ ml^{-1}$)	Seed type†	Incubation time (d)	$Pb(\mu g)\ddagger$ analysed	$MePb(\mu g)\S$ produced
10	I	7	6	8
1	I	7	12	15
0.1	I	7	~10	~13
1	II	14	82	106
1	II	7	80	103
		(+7)	(+10)	(+13)
1	II	7	56	72
		(+7)	(+20)	(+26)

*Starting concentration of $Pb(OAc)_2$ in a seeded 100-ml water sample (nutrient: glucose; seed type I or II) in a 250-ml gas wash bottle.

†Seeds were prepared from 10 ml A-water (— water from an aerated aquarium), 200 mg glucose and 10 mg urea, made up with water to 100 ml, and then incubated under N_2 . Seed type I: 10 ml seed (incubated for 2 weeks)+10 ml seed (incubated for 6 weeks)+10 ml A-water II: 10 ml seed (incubated for 5 d)+10 ml A-water.

‡'Volatile' Pb found in scrubber solution (see text).

§Amount of Me_4Pb equivalent to analysed amount of Pb.

||Same solution, additional incubation time in N_2 , after exchange of gas atmosphere, giving additional quantities of reaction product.

another source for Me_4Pb in the experiments with Me_3PbOAc (refs 1, 2). (The possibility of direct methylation of Me_3PbOAc still has to be investigated.) Furthermore one can expect that the portion of Me_4Pb chemically formed by redistribution is higher in sulphide systems², as the redistribution rate greatly increases with increasing concentration of added salt MX and with increasing polarisability of X^{3-4} . Also, since Pb^{2+} formed according to reactions (2) or (3) is precipitated as PbS , not enough Pb^{2+} is in solution to allow appreciable microbial alkylation to Me_4Pb .

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Muscle regeneration in dystrophic mice

In their letter¹, Hamburgh *et al.* set out to show that other workers in their field² had drawn incorrect conclusions from scanty experimental evidence. There are, however, certain inaccuracies in their text which do not do anything towards their claim of clarification.

In one paragraph we read that, "foetal cord from 15-d-old mouse embryos . . . were explanted". A little later we read, "spinal cords were dis-

sected from foetal mice aged 13–14 d *in utero*". This may seem a trivial point, but the foetal age is critical in these experiments, and such factual mistakes could lead other workers seriously astray.

Their most serious error is in the confusion of the two allelic mutant genes dy^2J and dy . Their experiments were carried out with the dy^2J mutant exclusively. They state, however, that, "normal muscle coupled with either normal or dy foetal spinal cord regenerated in culture". One can only assume that these authors were using 'dy' as an abbreviation for dystrophic, or else they are guilty of negligence. Whichever applies, it seriously detracts from the value of the work.

They have also misinterpreted my letter³ in which I did not confirm their work because my experimental system was entirely different. I did not say that "dystrophic" muscle would regenerate normally, since of the two mutants I described, only one (dy^2J) showed regeneration which was normal. It is obvious that little attention was paid to my concluding paragraph, which emphasised the need for caution and accuracy when working with murine muscular dystrophy.

With an air of finality the authors, to whom these criticisms are directed, concede that tissue culture may no longer be a fruitful research tool in this field.

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¹ Hamburgh, M., Peterson, E., Bornstein, M. B., and Kirk, C., *Nature*, **256**, 219–220 (1975).

² Gallup, B., and Dubowitz, V., *Nature*, **243**, 287–289 (1973).

³ Parsons, R., *Nature*, **251**, 621–622 (1974).

HAMBURGH ET AL. REPLY—We admit that the reference to foetal age may have been misleading! Timing of foetal age depends on the method of counting: some investigators call fertilisation, as shown by the appearance of the vaginal plug, day 0 whereas others call it day 1. It is always implied that any designation of foetal age is ± 12 h.

The designation 'dy' is an acceptable abbreviation for dystrophic and as the text clearly states that "phenotypically dystrophic mice were obtained from matings between tested homozygotes for the dystrophic gene dy^2J ", this should have been sufficient to clarify the point.

As for Parsons' letter² confirming our earlier work, he himself states² that "my results are essentially similar to those of Paul and of Hamburgh *et al.*". Although there may be a difference between "results that are essentially similar" and results that confirm, it is, however, so fine, that we admit, it escaped us. Parsons states⁴ that little attention was paid to his concluding paragraph² which emphasised the need for caution and accuracy when working with murine muscular dystrophy. We are fully familiar with the differential regenerative capacity exhibited by minced muscle obtained from the 129/ReJdy strains and the C57BL/6J dy^2J strains in his culture conditions.

Our comment that the tissue culture set up may not be as well suited as originally anticipated merely expresses an experience shared by other investigators that many genetic defects do not express themselves *in vitro*. Parsons interprets the sense of this comment to mean that "tissue culture may no longer be a fruitful research tool in this field".

We should like to take this opportunity to mention, however, that whatever the reasons for the different result, between the experiments by Gallup and Dubowitz² and our own¹, they may well be related to differential expressivity of the dy^2J gene. Slightly different tissue culture environments, differences in foetal age sex and other, yet to be identified factors, come to mind. Genes differ both in expressivity and penetrance in different environments, and gene mutations can often be revealed only by proper challenges. We consider Gallup and Dubowitz's series to be valid and most stimulating experiments.

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¹ Hamburgh, M., Peterson, E., Bornstein, M. K., and Kirk, C., *Nature*, **256**, 219–220 (1975).

² Parsons, R., *Nature*, **251**, 621–622 (1974).

³ Hamburgh, M., Bornstein, M. B., Peterson, E., Masurovsky, E. B., and Kirk, C., *Progr. Brain Res.*, **40**, 497–508 (1973).

⁴ Parsons, R., *Nature*, **259**, 158 (1976).

⁵ Gallup, B., and Dubowitz, V., *Nature*, **243**, 287–289 (1973).

Model ages

HUTCHISON *et al.*¹ have reported U–Pb analyses on samples of the meteorite Nakhla which confirm that this meteorite is younger than 3.68 Gyr. In both their title and their discussion there seems to be some apprehension about the ‘validity’ of model ages. Because of the considerable utility of the concept of model ages, we attempt here to clarify this matter.

Consider the number of atoms of a daughter nuclide (D) and of its radioactive parent (P) which are present in a sample today. Let us assume that a non-radiogenic isotope of the daughter nuclide is also present in the sample (the number of atoms being S). For a given reference ratio $(D/S)_M$, the model age (τ_M) of the sample is given by:

$$(D/S) = (D/S)_M + (P/S)(\exp(\lambda\tau_M) - 1)$$

τ_M is the time taken for the (D/S) ratio of the sample to evolve from the reference to the measured value if the system has remained closed. The trajectory in Fig. 1 leading to the present ratio (point A) must satisfy $D(\tau)/S(\tau) = (D/S) - (P/S)(\exp\lambda\tau) - 1$. Point B (where the real trajectory departs from the equation) represents the last time the system was

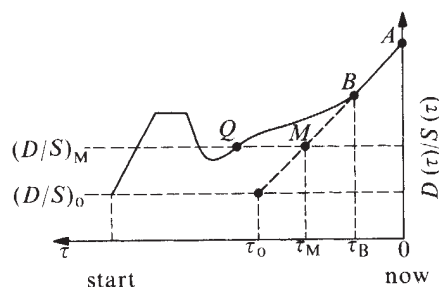


Fig. 1 Schematic diagram showing the evolution of $D(\tau)/S(\tau)$ with time for an open system. The value τ_0 yields the maximum age of the sample.

disturbed. The time when the system last passed through $(D/S)_M$ is at point Q . Estimates of the initial $(D/S)_M$ for Sr and Pb have been obtained from measurements in ancient samples with low values of P/S (refs 3–9). Designating these reference values as ‘0’ we have the corresponding model ages τ_0 given by $(D/S) = (D/S)_0 + (P/S)(\exp(\lambda\tau_0) - 1)$. If $(D/S)_0$ were the lowest possible ratio for solar system material, then the corresponding model age would be a strict upper limit to its age ($\tau_0 \geq \tau_B$) since $(D/S) - (D/S)_0$ is the largest change for a closed system which is now at (D/S) . For objects which have remained closed systems since the time of the hypothetical earliest planetary objects, τ_0 would be the ‘age of the Solar System’. It follows that samples which yield model ages τ_0 in the neighbourhood

of 4.6 Gyr may represent ancient materials with a relatively simple history. Values of τ_0 which are much greater or much less than ~ 4.6 Gyr constitute unequivocal evidence of open system behaviour at times less than 4.6 Gyr ago.

In general, the model age τ_0 must always be greater than the most recent time that chemical differentiation took place. For example, a devitrified glass from Nakhla yields a model age relative to ALL or BABI of $\tau_{ALL} \approx \tau_{BABI} = 1.78$ Gyr (ref. 2). It is thus evident that Nakhla has been subject to open system behaviour for Rb and Sr, at least on a microscopic scale, more recently than ~ 1.78 Gyr ago. The actual time of the last recrystallisation event was determined by a Rb–Sr internal isochron which gives an age of ~ 1.37 Gyr (ref. 2). In addition to the age of crystallisation or recrystallisation of Nakhla, we can inquire whether this event consisted of local element migration (for example) on a mm scale, of an ~ 4.6 Gyr-old object, or whether Nakhla formed by element mobilisation on a much coarser scale at times much younger than 4.6 Gyr. The Rb–Sr model ages for ‘total’ chips of Nakhla obtained from different meteorite fragments yield τ_{BABI} from 3.58–2.97 Gyr, and show that the ‘total’ meteorite was produced by differentiation at times strictly younger than 3.58 Gyr. The model age arguments given above are of course applicable to all parent–daughter systems. The data of Hutchison *et al.*¹ show that ‘total’ rock U–Pb model ages range from 4.10–3.68 Gyr, thus requiring a differentiation time younger than 3.68 Gyr. It is evident that all these data confirm a young differentiation age for the Nakhla parent body.

Based on petrographic features, it is most plausible to consider Nakhla as an igneous rock with a simple history. The Rb–Sr data prove that this object was subject to almost complete isotopic equilibrium 1.37 Gyr ago, and analyses of ‘total’ rocks show that the parent body was differentiated at least once at a time less than 3.6 Gyr ago. While the data define a rather good linear array, however, the precise age of crystallisation or recrystallisation is not exactly defined. The ‘total’ rocks do not lie on the isochron. Since it is reasonable to assume that the total rocks are comprised of their constituent mineral phases, it follows that some mineral phases in the rock must deviate even more widely from the linear array. The small but distinct deviations of the data from an isochron seem to require that the original differentiation which produced Nakhla occurred more than 1.37 Gyr ago, or that the deviations are the result of recent alterations or analytical difficulties.

In the preceding comments we have assumed that the initial values $(D/S)_0$ were well defined. This has been considered doubtful by Hutchison *et al.*¹. For the case of Sr, an extensive search for

primitive $^{87}\text{Sr}/^{86}\text{Sr}$ has been carried out on meteorites and lunar and terrestrial samples^{3,4,9,10}. Since the Rb/Sr ratio in the solar nebula is ~ 0.5 , the rate of evolution of $^{87}\text{Sr}/^{86}\text{Sr}$ corresponds to a change of 0.3% per 100 Myr. It is thus possible that a wide range in initial $^{87}\text{Sr}/^{86}\text{Sr}$ could be found in ancient Solar System objects if condensation and fractionation processes occurred for a time interval of $\sim 10^8$ yr. The lowest $^{87}\text{Sr}/^{86}\text{Sr}$ value so far observed is, however, below BABI by only three parts in 10^4 which will not significantly alter the model ages for Nakhla. With regard to the U–Pb system, the basic data as first reported by Patterson *et al.*⁵ have been modified over the years but the essential results are not changed^{6–8}. If we consider $^{238}\text{U}/^{204}\text{Pb} \sim 0.3$ in carbonaceous meteorites^{7,8,11–13} as representative of the solar nebula, then we calculate a change in $^{207}\text{Pb}/^{204}\text{Pb}$ at 4.6 Gyr of 0.2% per 100 Myr. This rate is similar to that for $^{87}\text{Sr}/^{86}\text{Sr}$; however, in contrast to the case of Sr, the rate of evolution of Pb in the solar nebula is much less than that on the Earth, Moon and many meteorites, reflecting strong U/Pb enrichment for terrestrial-type planets in the early Solar System. It is possible that the iron meteorites studied for initial Pb were produced by planetary differentiation of a body with high $^{238}\text{U}/^{204}\text{Pb}$ and could reflect rapidly evolving Pb compositions. The general agreement of troilite Pb with that found in carbonaceous chondrites, which are certainly not planetary differentiates, would, however, seem to exclude the possibility of the large shifts in the value of primordial Pb referred to by Hutchison *et al.*¹.

We conclude that model ages provide an upper limit to the age of any system and that total rock model ages provide an insight into the differentiation processes active in planetary evolution. For Nakhla, we conclude that all methods confirm a young formation age for this meteorite, that some complexities are manifest in the detailed history of this object and finally, that no information about primitive initial isotopic compositions can be deduced from data on this young body.

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HUTCHISON, GALE, AND ARDEN REPLY—We are grateful to Wasserburg and Papanastassiou for their courteous and clear discussion¹ of model 'ages' arising from our paper² on some preliminary U-Pb data for the achondritic meteorite Nakhla.

We agree that a single-stage model 'age' computed from a primitive Solar System daughter/stable nuclide ratio (D/S)₀ defines the oldest limit for the most recent time at which the particular system became closed. This is stated in our paper on the Rb-Sr chronology of Nakhla³. We agree also that it is likely that model 'ages' greatly different from 4.6 Gyr constitute evidence for multiple stage open system evolution. This has already been stated by one of us⁴. We feel, however, that it is possible that the ordinarily accepted 'primitive' Solar System initial isotopic compositions might not apply to all objects in the Solar System. In our U-Pb studies we have had several indications that they are not universally applicable. Recently discovered anomalous ratios of two light elements⁷ suggest that we may still have something to learn about isotopic abundances in the early Solar System.

It is still our view that model 'ages' should not be used without a clear statement of their meaning, as they are readily confused with true ages by those who are unfamiliar with the field of geochronology. Further, when only a limited number of model ages are available, the upper limit set may be so far from the true age as to be misleading. An example is provided by our own Rb-Sr work on Nakhla³, where 15 Rb-Sr determinations on diopside, plagioclase, olivine and various magnetically separated mixtures, yield τ_{BABI} model ages ranging from 5.9 to 2.33 Gyr, whereas the internal isochron yields a true age of 1.24 Gyr.

Our data indicate that the Nakhla meteorite crystallised from a melt precisely 1.24 ± 0.01 Gyr ago³ (not 1.37 Gyr ago⁵) with complete isotopic mixing. Because the U-Pb data demand a history of three (or more) stages (unless unusually low initial Pb isotope ratios are invoked) it is almost certain that a large scale chemical fractionation occurred at that time. This age is, of course, less than the whole meteorite model 'ages'. We reiterate that such a fractionation would have obliterated evidence of the earlier history

of the parent body of Nakhla. 'Gross differentiation' of the parent body (ref. 5, abstract) could, therefore, have occurred before 3.6 Gyr, if one assumes that the later, 1.24-Gyr, event was due to volcanism or impact melting on a less than planetary scale, as is discussed in more detail in ref. 3. Wasserburg and Papanastassiou¹ correctly argue that Rb-Sr and U-Pb model 'ages' for "total" meteorite chips require a differentiation age for the total meteorite strictly younger than 3.58 or 3.68 Gyr respectively (in fact this time is 1.24 Gyr), but incorrectly assume that this argument may certainly be extended to the parent body of Nakhla as a whole.

The presence in Nakhla of water-bearing silicate has now been confirmed, but in addition, evidence of mild, post-crystallisation shock was found⁶. Presumably it was during this mild shock event that the Rb-Sr system was disturbed on a millimetre scale, as shown by the failure of meteorite chip data to lie on the mineral isochron. There is no longer a need to invoke either redistribution of Rb during or after atmospheric flight³, or incomplete homogenisation of Sr 1.37 Gyr ago during dry metamorphism⁵. We are pleased that Wasserburg and Papanastassiou now prefer to agree with us in interpreting Nakhla as an "igneous rock with a simple history".

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- ¹ Wasserburg, G. J., and Papanastassiou, D. A., *Nature*, **259**, 159-160 (1976).
² Hutchison, R., Gale, N. H., and Arden, J. W., *Nature*, **254**, 678 (1975).
³ Gale, N. H., Arden, J. W., and Hutchison, R., *Earth planet. Sci. Lett.*, **26**, 195 (1975).
⁴ Gale, N. H., *Earth planet. Sci. Lett.*, **17**, 65 (1972).
⁵ Papanastassiou, D. A., and Wasserburg, G. J., *Geophys. Res. Lett.*, **1**, 23 (1974).
⁶ Ashworth, J. R., and Hutchison, R., *Nature*, **256**, 714 (1975).
⁷ Lee, T., and Papanastassiou, D. A., *Geophys. Res. Lett.*, **1**, 225 (1974).

North Sea phytoplankton

REID¹ described large scale changes in North Sea phytoplankton communities which have been detected since the mid-1960s by the Continuous Plankton Recorder survey. These changes were characterised by a marked decline in diatom populations, a possible increase in microflagellate abundance, and a decline in the zooplankton biomass. Reid concluded that eutrophication of North Sea waters and climatic changes could not, by themselves, easily explain these alterations in plankton communities.

I suggest that another set of factors, namely the presence of persistent industrial pollutants in the North Sea²,

may partially account for these changes. Many species of marine phytoplankton, particularly diatom species, show sensitivity to low levels of industrial waste products, including heavy metals³, petroleum hydrocarbons⁴, and stable chlorinated hydrocarbons such as polychlorinated biphenyls⁵. Alterations in the species composition of phytoplankton communities through selective toxicity of pollutants have been observed in the laboratory⁵ and in freshwater ponds⁶. The predictions, based on laboratory research, suggesting that pollution-linked alterations in the species composition of phytoplankton communities would result in disturbances of zooplankton communities⁷ (due to selective herbivory) are consistent with the observations described by Reid¹. The degree to which the North Sea plankton are exposed to industrial wastes is subject to further study, though plankton in neighbouring waters are highly contaminated with organic pollutants^{7,8}. (It is interesting that sublethal concentrations of pollutants can interact with various environmental factors to enhance pollutant toxicity to marine organisms, including phytoplankton.) Furthermore, North Sea animals higher in the food chain are heavily contaminated with industrial waste products⁹, indicating their general presence in the resident biota.

Longhurst *et al.*¹⁰, citing the natural variability of marine populations, commented that changes in oceanic plankton communities often cannot clearly be traced to marine pollution. Indeed, it is difficult to pinpoint any single environmental factor as the sole cause of community disruptions, because so many variables may interact to affect biological systems. I propose that the presence of persistent pollutants be considered one of the possible contributing factors influencing the plankton communities in the North Sea.

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- ¹ Reid, P. C., *Nature*, **257**, 217-219 (1975).
² Cole, H. A., in *Marine Pollution and Sea Life* (edit. by Ruivo, M.), 3-10 (Fishing News Books Ltd, London, 1972).
³ North, W. J., Stephens, G. C., and North, B. B., *ibid.*, 330-340.
⁴ Mironov, O. G., *ibid.*, 222-224.
⁵ Fisher, N. S., Carpenter, E. J., Remsen, C. C., and Wurster, C. F., *Microb. Ecol.*, **1**, 39-50 (1974).
⁶ Hurlbert, S., Mulla, M. S., and Willson, H. R., *Ecol. Monogr.*, **42**, 269-299 (1972).
⁷ Linko, R. R., Rantamaki, P., and Urpo, K., *Bull. environ. Contam. Toxic.*, **12**, 733-738 (1974).
⁸ Williams, A., and Holden, A. V., *Mar. Poll. Bull.*, **4**, 109-111 (1973).
⁹ Holden, A. V., in *Marine Pollution and Sea Life* (edit. by Ruivo, M.), 266-272 (Fishing News Books Ltd, London, 1972).
¹⁰ Longhurst, A., *et al.*, *New Scientist*, **54**, 500-502 (1972).

reviews

THIS book* gives an account of the famous encounter between Lord Kelvin and the geological establishment over the use of physical arguments to set limits to the age of the Earth. It was an epic struggle which continued for 50 years with great vehemence and some acrimony.

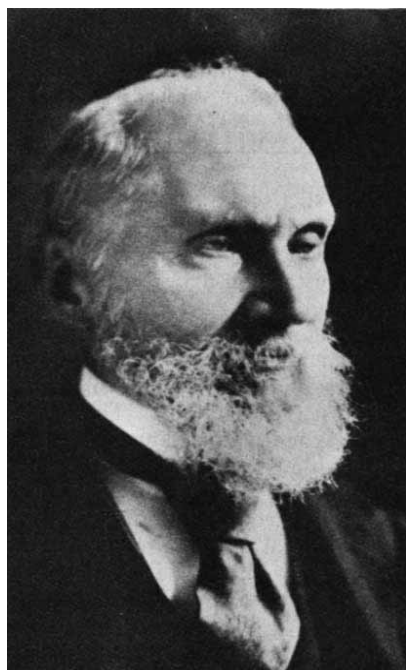
In their thoughts about time the geologists of the middle of the nineteenth century were not much inclined to arithmetic. They had inherited from Hutton and Lyell the idea that the Earth showed "no vestige of a beginning, no prospect of an end". This and the related ideas, that things have always been much as they are now, and that causes at present at work could, given enough time, account for all that has ever happened on earth, were the basis of their science and, in large degree, still are.

Very early in his career Kelvin became interested in the time scale of the Earth. His two principal methods of setting an upper limit were the calculation of the time for which the earth's original heat could supply the outward flow observed at the surface and a similar calculation for the sun. Both calculations required few and very plausible assumptions. Kelvin, and most physicists of the time, had great confidence in the results.

Kelvin's first estimate of 100–400 Myr did not greatly alarm the geologists. They no doubt knew that 100 Myr was a finite time, but it was so large a number that it seemed unnecessary to quarrel with it. Unfortunately, as he grew older, Kelvin grew more parsimonious of time and reduced his estimate to 20–30 Myr with threats of further restriction. This was too much, or rather too little; the few geologists who had disliked the 100 Myr limit were joined by the majority of their colleagues and a classic encounter occurred between two groups with almost no ideas in common. Kelvin had the prestige of physics, of Newton and of the divinely prescribed order of Nature behind him. Clearly he could not be wrong. The geologists had gut feelings that any specified length of time was inadequate, but little pos-

sibility of convincing others. Darwin, for example, estimated the time necessary to excavate the Weald of Kent as 400 Myr, but had no real basis for his statement, which he came greatly to regret.

Epic struggle with geological establishment



Edward Bullard

The story of the collision of two such disparate groups is admirably told in this book. Besides the main contestants all kinds of fascinating figures flit across the scene, including Lord Salisbury (the Prime Minister), Clarence King (the explorer of Colorado and Utah), T. J. J. See (the presumed author of that once celebrated work *The Life and Remarkable Discoveries of T. J. J. See*) and even, briefly, Frank Harris (the author provides a reference to his *Life and Loves*). The course of the dispute is

set out in detail and is of great interest to anyone concerned with the development of ideas about the Earth. Here the ideas of stratigraphy, evolution and physiography first met theoretical physics. The interest is, of course, enhanced by the fact that the biologists and geologists were right in saying that 30 Myr was not enough time, even though they had no way of providing convincing numerical estimates; how could one say how many years it took to convert *Eohippus* into a Derby winner?

The debacle induced by the discovery of radioactivity is well described; one of the oddest features is that no one seems to have been much concerned that, for the next 30 years, there was still no convincing account of the source of the Sun's heat.

In spite of the careful account of all these things some readers may feel a little deprived. An historian must study the past in its own terms. He must not ask 'What was Henry VIII's attitude to women's lib?'. This viewpoint has been adopted also by historians of science and, in some degree, is clearly necessary. But should one go the whole way? The author of this book consistently refrains from saying that anything is right or wrong, silly or unjustified. If Kelvin assumes that the Earth was initially at 1,500 °C, the author reports that he said it and that it was a more or less arbitrary choice, but he does not add that it matters very little what figure is taken. On the other hand no one would guess that Kelvin's assumption of the absence of convection within the Earth was crucial and that the attacks on it were well based. The history of science is different from other kinds of history; there is the additional fact that some things are correct and some wrong *sub specie aeternitatis*. The reasons for the beliefs are a function of the surrounding culture, but being right or wrong is not a thing of one age alone. In the study of the motions of the planets Descartes was wrong and Newton was right, and so it will always be. It is part of the function of the historian to say how and why. We and he lose a good deal if he backs away from the question. There were geniuses, inspired guessers, cranks, stick-in-the-muds and rash men in the past, just as there are today, and it is a pity to treat them all alike and leave the reader to decide which is which. □

**Lord Kelvin and the Age of the Earth*. By Joe D. Burchfield. Pp. xii+260. (Macmillan: London and Basingstoke, July 1975). £10.

Metallic catalysts

Structure of Metallic Catalysts. By J. R. Anderson. Pp. ix+469. (Academic: New York and London, June 1975.) £12.60; \$33.75.

THE technical importance of catalysts is such that an understanding of their structure is of obvious importance, whether from the view-point of the extent of surface accessible to reactants, or of the more intimate details of surface topography, chemistry, or electronic structure which might influence adsorption processes. Also, in the case of supported catalysts, the structure of the substrate can determine the state of dispersion of the metal and perhaps contribute to the overall reaction. This book is the first attempt to bring together information in these areas from the plethora of published literature, and from this aspect alone is a considerable achievement.

Available information on the structure of typical metallic phase catalysts is covered in some detail, particularly that relating to model systems where

surface contamination can be more carefully controlled and which can therefore be used as standards for comparison. There are chapters on supports, massive, and dispersed, metal catalysts, and the structure and properties of small metallic particles. The book concludes with an outline of some appropriate techniques for the physical and chemical investigation of such structures, and there is an appendix which describes the preparation of some examples of typical metallic catalysts.

The state and scope of the subject dictate that the work be largely descriptive and of a review nature. It is, however, well and critically written, acceptably illustrated, and generally readable. It should make an admirable starting point for those entering the fields of catalysis or surface chemistry; the wealth of detail, some of it in useful tabular form, and the large number of quoted references, unfortunately without an author index, will also make it a useful addition to the libraries of any academic or industrial laboratory engaged in similar studies.

G. K. L. Cranstoun

Hydro, azo, azoxy

The Chemistry of the Hydrazo, Azo and Azoxy Groups. Edited by Saul Patai. Part 1: Pp. xiv+1-598. Part 2: Pp. xiv+599-1,190. (Wiley/Interscience: London and New York, September 1975.) £17 each part; £33 two volume set.

THIS is the sixteenth volume to appear in Professor Saul Patai's monumental series on the chemistry of individual functional groups; there are five volumes to come. There is no detailed consideration of azo dyes on grounds of space, and diazonium salts and diazoalkanes are to be covered in a subsequent volume.

The general style, arrangement and coverage closely parallel those of previous volumes, including the usual articles on structure, thermochemistry, electrochemistry, photochemistry, radiation chemistry, mass spectra, basicity and complexing, of the three groups. There are also interesting accounts of chiroptical properties, directing and activating effects, biological formation and reactions, syntheses and uses of isotopically labelled species, rearrangements (a particularly good and interesting contribution), ionic reactions, and radical reactions. There is a useful chapter on preparative procedures, and also two interesting longer chapters on oxidation and synthetic uses, and reduction and synthetic uses, of the groups. Finally there are more specific, and specialised items on transition metal chemistry of the groups, on conformational analysis of hydrazines, and a long but very interesting chapter on the formation and fragmentation of cyclic azo compounds.

The literature seems in general to have been surveyed to the end of 1973—I could find only fourteen references, out of many hundreds, dated 1974—although this datum line does not seem to be uniform for all articles. The subject index could usefully have been a little more comprehensive, perhaps at the expense of the author index that one would have thought to be relatively less important. The general printing and layout of the reaction schemes is markedly superior to that in most of the previous volumes. **Peter Sykes**

Opiate receptors

Opiate Receptor Mechanisms: Neurochemical and Neurophysical Processes in Opiate Drug Action and Addiction. (Based on a work session of the Neurosciences Research Program.) By Solomon H. Snyder and Steven Matthyse. Pp. 116+vi. (MIT Press: Cambridge, Massachusetts and London, June 1975.) \$8.95.

THIS book is a record of a Work Session of the Neurosciences Research Program sponsored by the Massachusetts Institute of Technology and held in Boston during May 1974. The symposium was an unqualified success because it came at a time when the work of several groups, particularly those of A. Goldstein, L. Terenius, S. H. Snyder and C. B. Pert, and E. J. Simon, had greatly contributed to our knowledge of the nature and distribution of opiate receptors.

When the symposium was planned, various lines of indirect evidence had accumulated which suggested that there were in the central nervous system endogenous ligands that interacted with the opiate receptors. Evidence for the existence of such ligands was presented by L. Terenius, and by J. Hughes and H. W. Kosterlitz. G. W. Pasternak and S. H. Snyder mentioned supporting evidence which was updated in the published version.

These two sections are probably the most topical parts of the book. In the introduction, there is a brief review of the chemistry and action of opiates for readers unfamiliar with the field. The second chapter of the book deals with the biochemical identification of opiate receptors and the third with agonist-antagonist interactions. The fourth considers neural mechanisms and contains a discussion by D. J. Mayer of the interesting experiments on analgesia induced by electrical stimulation of the periaqueductal grey matter and its prevention by the opiate antagonist, naloxone. The fifth chapter deals with biochemical phenomena in opiate action and addiction, the sixth with addiction, the seventh with the discussion review on further research. The final chapter is a stimulating and provocative essay by S. H. Snyder on a model of opiate receptor function with implications for a theory of addiction.

This is a well-produced book presenting expertly and critically the state of our knowledge in an important field. Because the various chapters are not written by the individual authors but by the editors with the help of a small number of participants, the style is uniform and pleasing. It is unavoidable that part of the book has already been overtaken by events in a field in which a considerable number of workers are in constant and vigorous competition with each other.

H. W. Kosterlitz

Erratum. Gordon and Breach, publishers of *Composition of Cosmic Radiation* by Apparao, reviewed jointly with another book on the same subject (*Nature*, 258, November 6, page 40), point out that the reviewer's statement about the comparative prices per page of the two books is incorrect. The book by Apparao has, in fact, the lower price per page. We apologise for the error.

Interfacial Phenomena in Metals and Alloys. By Lawrence E. Murr. Pp. xiv+37. (Addison-Wesley: Reading, Massachusetts, and London, September 1975.) Cloth \$24.50; Paper \$14.50.

THE five chapters of this book make up a comprehensive treatment of those aspects of surface science which contribute to the properties of materials, and in particular to metals and alloys. The first is concerned with surface thermodynamics of solid surfaces, starting from Gibbs' model, but including a discussion of the distinction between surface stress and surface tension, and the effect of vacancies and other singularities of structure. In the second chapter these principles are applied to the description of conditions for the establishment of interfacial equilibria, especially the evaluation of interfacial free energy ratios. Because of the crucial importance of interfacial free energy values in determining the forms of crystals and grains, chapter 3 is concerned with their determination in different systems. The last two chapters treat the structure and properties of interfaces respectively.

A particular feature of this book is the wealth of factual information which has been gathered into tables of surface and interfacial free energies of solids, and related quantities, occupying almost 10% of the available space. Taken with over 800 references, this ensures that the book will be extremely useful as a source of critically appraised facts for research workers in addition to its primary function of instructing students of materials science in important principles, and exercising them with fifty problems, mostly numerical. Comprehensive author and subject indexes have been provided, and the book is very clearly printed and copiously illustrated. **A. Couper**

Organoborane Chemistry (Organometallic Chemistry: Series of Monographs.) By Thomas Onak. Pp. x+360. (Academic: New York, San Francisco and London, July 1975.) \$38.00; £18.25.

THIS book is one of an important series on organometallic chemistry, edited by P. M. Maitlis, F. G. A. Stone and R. West. The topic of organoborane chemistry has developed very rapidly: this is the first review for almost ten years dealing explicitly with compounds having boron-carbon bonds, although others are available

on related topics, such as hydroboration. Excluded are other organic boron compounds such as boron esters.

A brief introduction (1 page) is followed by Chapter 2 (18 pages), concerned mainly with X-ray, spectroscopic, and thermodynamic data. Chapter 3 (116 pages) deals with methods of B-C bond formation and cleavage. [For those familiar with the field it will be clear that the author has used a very similar presentation to that used in my article in Muetterties' monograph of 1967.] Chapters 4-6 (80 pages), on four-coordinate organoboranes, organodiboranes, and other polyboranes, are particularly welcome.

Books brief

There are nearly two thousand references which include papers published in 1973. Inevitably, therefore, the discussion is selective, particularly as about a quarter of the 224 pages of text is taken up by tables. Nevertheless, the monograph is informative and well-written and should be essential reading for anyone active in this field. **M. F. Lappert**

Interactions on Metal Surfaces. (Topics in Applied Physics, Volume 4.) Edited by R. Gomer. Pp. xi+310. (Springer: Berlin and New York, 1975.) DM78; \$33.60.

THIS book contains seven articles on selected aspects of the chemisorption of gases on metals. A model (chapter 1) consisting of an alternating potential within the metal and a discontinuity at the surface enables the calculation of wave functions near the surface using generalised Wannier functions. The electron work function is then calculated from a continuum model into which the effect of crystallinity is introduced as a perturbation, and adsorption as localised screening. The chemisorption bond (chapter 2) is described initially in terms of an LCAO-MO model, which is extended to include overlap between metal and adsorbate states and lateral interactions.

Four chapters then give an account of experimental aspects of chemisorption, the first summarising studies of

clean surfaces and adsorption using a variety of techniques, whereas the other three discuss thermal and electron impact desorption, photoemission and field emission spectroscopy, and low energy electron diffraction. The final chapter on heterogeneous catalysis is included to indicate, in the editor's words, "that there is more to interactions on surfaces than the adsorption of hydrogen on the (100) plane of tungsten".

There is some overlap in subject matter between the chapters, but each presents its author's individual view of recent advances. There are over 700 references, a useful index and many excellent illustrations. It is clearly not a 'first text-book' of chemisorption, but research workers will find it both stimulating and informative. **A. Couper**

Genetics and Psychopharmacology. (Modern Problems of Pharmacopsychiatry, Volume 10.) Edited by J. Mendelewicz. Pp. viii+132. (Karger: Basel, London and New York, 1975.) Sfr.58; DM55; \$24.25.

PSYCHIATRY relies on a wide range of investigational techniques ranging from the psychoanalytical to the biological. Among the latter, genetic studies have suggested that many major psychiatric illnesses have a hereditary component. The introduction of effective drugs for these illnesses has emphasised the importance of biological factors in the causation and mechanism of psychiatric illnesses. In this book, the two threads are drawn together. The points of contact, however, remain few and one or two of the contributions in this multi-author book contain little or no genetics. The chapters are of a generally high standard and range widely from the role of heredity in alcoholism to the genetic mechanisms governing the rate of acetylation of the monoamine oxidase inhibitor, phenelzine. Monoamine oxidase is the main interest of almost half the book but claims that levels of this enzyme in blood platelets are abnormal in patients with depression and schizophrenia and in relatives of the latter seem premature. Brain levels (surely more relevant) are quite normal in psychiatric patients. One is left wondering whether the greatest difficulties in biological psychiatry relate not to the biological techniques but to the unsystematic way in which diagnoses are made and symptoms assessed. **M. H. Lader**

obituary

The malariologist **Meir Yoeli** died suddenly on December 5, 1975 in New York. He was born in Lithuania in 1912 and graduated with an M.Sc. from the University of Kaunas in 1934. Soon afterwards, he fled to Palestine and was appointed a lecturer at the Hebrew University of Jerusalem in the department of tropical medicine. Here he was introduced to the subject of malaria by two great masters—Gideon Mer and Saul Adler F.R.S., and continued this work in 1938, in a year's study with Missiroli and Angelini in Italy. Yoeli received his MD at Basel in 1939, and served under Colonel George Macdonald as a malariologist with the R.A.M.C. in World War II. Some years after the war, he left Israel for the United States of America, where he obtained an appointment in the Department of Preventive Medicine of the University of New York, and where he continued to work until his death.

Yoeli is best known for his research in rodent malaria. He had set his heart on solving the problem

of the life cycle of these parasites—a problem which had defeated many other investigators. He first succeeded in transmitting *Plasmodium berghei* through the mosquito in 1951 at the London School of Hygiene and Tropical Medicine, but it was another 13 years before he discovered the clue for producing viable infective forms (sporozoites): the secret of success lay in dropping to 19 °C, the ambient temperature in which his infected mosquitoes were kept—the same temperature at which natural transmission occurs in the cool highland forests of Zaire. This unexpected result was followed by another, equally spectacular—the rapidity of the exo-erythrocytic cycle of the sporozoite in the rodent's liver (2 d instead of the usual 8 d in primate malaria). Much of this work was done in collaboration with his Belgian friend—the late Professor Ignace Vincke—who was the original discoverer of rodent malaria in 1948. Yoeli extended his research to the immunological aspects of the infection, and recently made important contri-

butions to the aetiology of leukaemia.

Yoeli was as interested in art as he was in science. In 1974 he became an honorary member of the Hellenic society for the History of Medicine, and only his premature death prevented a compilation of his Hebrew poetry, literature and philosophy for publication. He will be greatly missed by his family and his friends all over the World.

P. C. G. Garnham

Dr James Linzell died on December 28, at the age of 54. After qualifying and working as a vet, he obtained his Ph.D. at Edinburgh in 1951. He spent the rest of his career in the Institute of Animal Physiology in Cambridge, where he became the Head of the Department of Physiology.

His major interest was the physiology of the mammary glands of farm animals, and his discoveries about the mechanisms for the starting of, supply to and production by these organs underpin the present understanding of the subject.

announcements

International meetings

March 1–4, **Breast feeding and the mother**, London, The CIBA Foundation, 41 Portland Place, London W1N 4BN, UK).

March 1–5, **Tunnelling '76**, London (The Secretary, Institution of Mining and Metallurgy, 44 Portland Place, London W1N 4BR, UK).

March 1–5, **Oilseed and vegetable oil processing technology**, Amsterdam (World Conference, AOCS, 508 S. Sixth Street, Champaign, Illinois, 61820).

March 1–5, **Analytical chemistry and applied spectroscopy**, Cleveland (Dan P. Manka, Program Chairman 1976, Jones and Laughlin Steel Corporation, Graham Research Laboratory, 900 Agnew Road, Pittsburgh, Pennsylvania 15230).

March 2, **Applications of linear free energy relationships in biological systems**, London (The Assistant Secretary, Society of Chemical Industry, 14 Belgrave Square, London SW1X 8PS).

March 2–3, **The working diver**, Colum-

bus, Ohio (Debra Klamforth, Battelle, Columbus Laboratories, 505 King Avenue, Columbus, Ohio 43201).

March 9–13, **Physics in industry**, Dublin (Eon O'Mongain, Organising Secretary, Physics Department, University College, Dublin, Ireland).

March 15–19, **Third international conference on culture collections**, Bombay (Professor F. Fernandes, Chairman, Local Organising Committee, Bombay University Club House, B. Road, Churchgate Reclamation, Bombay 400020, India).

March 17–18, **The semi-arid areas of the world**, London (The Executive Secretary, The Royal Society, 16 Carlton House Terrace, London SW1Y 5A9, UK).

March 22–23, **Information for the water industry**, Reading, UK (The Conference Organiser, Water Research Centre, Medmenham Laboratory, PO Box 16, Ferry Lane, Medmenham, Marlow, Bucks SL7 2HD, UK).

March 22–23, **Genetic engineering**, Glasgow (Dr P. J. Goddard, Organising Secretary, Nucleotide Group

Meeting, Department of Biochemistry, University of Glasgow, Glasgow G12 8OO, UK).

March 23–26, **Fundamental and applied dosimetry**, Saclay, France (M. Y. Le Gallic, Secrétaire général du VIII^{ème} Congrès International de la S.F.R.P., C.E.N. Saclay, B.P. n° 2,91190, Gif sur Yvette, France).

March 25–26, **The controversy about sweeteners**, Washington, D.C. (Barbara Jorgensen, NAS Office of Information, 2101 Constitution Avenue, N.W., Washington, D.C. 20418).

March 26, **Fish farming**, Edinburgh (The Executive Secretary, The Royal Society of Edinburgh, UK).

March 28–April 1, **International symposium on fluorescein angiography**, Ghent (Secretariat, c/o Holland Organising Centre, 16 Lange Voorhout, The Hague, The Netherlands).

March 29–April 1, **International symposium on urinary stone formation**, Davos, Switzerland (Professor H. Fleisch, Department of Pathophysiology, Murtenstrasse 35, CH- 3008 Bern, Switzerland).